ANTIOXIDANT ENHANCEMENT OF THERAPY FOR HYPERPROLIFERATIVE CONDITIONS

This application is a continuation application of U.S.S.N. 08/967,492, entitled "Antioxidant Enhancement of Therapy for Hyperproliferative Condtions," filed on November 11, 1997, by Rebecca Chinery, R. Daniel Beauchamp, Robert J. Coffey, Russell M. Medford and Brian Wadsinski, and converted to a provisional application by a petition filed on June 30, 1998; which is a continuation-in-part application of U.S.S.N. 08/886,653, entitled "Antioxidant Enhancement of Anti-Neoplastic Therapy," filed on July 1, 1997, by Rebecca Chinery, R. Daniel Beauchamp, and Robert J. Coffey, and converted to a provisional application by a petition filed on June 30, 1998.

Field of the Invention

This invention is in the field of medicinal chemistry, and more specifically, describes methods and compositions for antioxidant enhancement of therapy for hyperproliferative conditions.

BACKGROUND OF THE INVENTION

A wide range of disorders involve the hyperproliferation of cells, ranging from psoriasis to benign and malignant tumors. These disorders are generally caused by a

loss of control over normal cell growth, differentiation, or the process of programmed cell death (apoptosis). Many of the abnormalities that underlie these disorders, particularly cancer, occur at the genetic level. Antineoplastic agents (also known as cytotoxic agents) are often used in the treatment of hyperproliferative conditions.

5   Therapy with antineoplastic agents is successful in the treatment of a number of malignant conditions; however, in most it is used to palliate the symptoms and to prolong life in patients with advanced disease. Two groups of drugs used in the treatment of hyperproliferative conditions are antimetabolites and alkylating agents. Antimetabolites can be subdivided into folic acid, purine, and pyrimidine derivatives.

10   In addition, several natural products, or their derivatives, have been used as mitotic inhibitors. These include vinca alkaloids, and the derivatives of podophyllotoxin. There remains an effort in the research community to understand the genetic basis for abnormal cell hyperproliferation, which can vary among its various manifestations, and to develop therapeutic methods to successfully treat these serious conditions.

15   For about four decades, the antimetabolite 5-fluorouracil (5-FU), and nucleosides which include this base (e.g., 5-fluoro-2'-deoxyuridine or FdUrd), have remained among the few "standard" drugs effective against solid tumors in man. 5-Fluorouracil is used mainly for the treatment of colorectal, ovarian, renal, breast and head and neck cancers. 5-Fluoro-2'-deoxyuridine is used for the treatment of solid

20   tumors, including hepatic metastases of advanced gastrointestinal adenocarcinomas, renal cell carcinomas, advanced ovarian cancer, and squamous cell carcinomas of the head and neck. The clinical utility of the fluoropyrimidines is limited by the host-

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toxicity induced by the administration of these compounds. Manifestations of the host-toxicity of the fluoropyrimidines include mainly gastrointestinal epithelial ulceration, myelosuppression and, to a lesser extent, cardiotoxicities, hepatotoxicities and neurotoxicities.

5   A population of cancer patients is intolerant to treatment with 5-fluorouracil and 5-fluoro-2'-deoxyuridine. The intolerance to 5-fluorouracil was initially attributed to a deficiency or low activity of dihydrouracil dehydrogenase (DHUDase, EC 1.3.1.2), the first enzyme in the catabolic pathway of 5-fluorouracil. However, it appeared that not all intolerant patients showed reduced dihydrouracil dehydrogenase

10   activities. Moreover, it has also been shown that cancers, treated with fluoropyrimidines, become resistant, i.e., develop tolerance towards these drugs.

          Colorectal cancer (CRC) is a multi-step process resulting from the accumulation of mutations in clonal populations of colonocytes. Mutations of the p53 tumor suppressor gene are a relatively late, yet common event in the pathogenesis of

15   colorectal cancer, occurring in over 80% of late adenomas and carcinomas (Fearon, et al., FASEB J. 6, 2789 (1992); Srivastarva, et al., *Contemp. Oncol.* April 63 (192); Kline, et al., *Cancer* (Phila. 73, 28 (1994). Conventional therapy for advanced disease, such as cytotoxic chemotherapy and gamma-irradiation, induce DNA damage in proliferating cells. This damage, through undefined mechanism(s), signals the

20   induction of p53, which, in turn, leads to inhibition of cellular proliferation by induction of G_1 cell cycle arrest and, in some instances, apoptosis. Thus, tumors lacking functional p53 are frequently refractory to such therapies (S.C. Righetti *et al.*,

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Cancer Res. 56, 689 (1996); J. S. Kovack *et al., Proc. Natl. Acad, Sci.* U.S.A. 93, 1093 (1996)), emphasizing the importance of developing treatments for advanced colorectal cancer that do not rely on functional p53.

The most effective single chemotherapeutic agent for advanced colorectal cancer to date remains 5-FU. The active metabolite of 5-FU, 5-fluorodeoxyuridine-5'-monophosphate (FdUMP), forms a complex with thymidylate synthase (TS) in the presence of reduced folate, thereby inhibiting enzyme activity, and depleting precursors for DNA synthesis. 5-FU is also incorporated into RNA, altering its processing and function, although how this correlates with cytotoxicity is unknown. Previous data suggest that 5-FU can utilize both p53-dependent and independent pathways (Pritchard, et al., *Pharmacol. Ther.* 72, 149 (1996)), although a loss of p53 function dramatically reduces 5-FU efficacy (B. Cohen *et al., Cancer* (Phila.) 67, 1859 (1991); Advanced Cancer Meta-Analysis Project, *J. Clin. Oncol.* 10, 896 (1992)).

In view of the lack of successful treatments for many hyperproliferative conditions, it would be of benefit both to identify important biological pathways which mediate the loss of normal cell function, including programmed cell death (i.e., apoptosis), and to identify compositions and methods for the treatment of these disorders.

U.S. Patent Nos. 5,035,878 and 5,294,430 disclose that dithiocarbamates can reverse the damage to the blood-forming function of the bone marrow (myelosuppression) caused by treatment with antineoplastic agents.

It is therefore an object of the invention to provide a method and composition for the treatment of abnormal cell proliferative conditions, including benign and malignant tumors.

It is another object of the present invention to provide a method and composition for the treatment of colon cancer.

It is a further object of the present invention to provide a method and composition for the treatment of solid tumors.

It is yet another object of the present invention to provide a method and composition for the treatment of diffuse tumors.

SUMMARY OF THE INVENTION

It has been discovered that antioxidants, including those specifically disclosed herein, induce cell cycle arrest (G1, G2, S and M type), and thus are useful to enhance the efficacy of antineoplastic drugs for the treatment of abnormal cell proliferation.

In one embodiment, therefore, the invention is a method to enhance the cytotoxic activity of an antineoplastic drug comprising administering an effective amount of the antineoplastic drug to a host exhibiting abnormal cell proliferation in combination with an effective cytotoxicity-increasing amount of an antioxidant.

It has further been discovered that antioxidants not only induce cell cycle arrest, but can cause the cell to maintain the state of arrest, and perhaps, induce apoptosis, by inhibiting enzymes which would otherwise turn off the process of cell cycle arrest.

Importantly, it has also been discovered that not only do antioxidants increase the cytotoxicity of antineoplastic agents to abnormally proliferating cells, they also decrease the toxicity of antineoplastic agents to normal cells. Therefore, antioxidants both increase the effectiveness and decrease the toxicity of antineoplastic agents. The palliative effect on normal cells is pronounced in epithelial cells. It has, in particular, been discovered that antioxidants exhibit this effect on cells other than white blood cells, as previously reported by Borch in U.S. Patent Nos. 5,035,878 and 5,294,430.

Thererfore, the invention includes a method to decrease the toxicity of an antineoplastic agent administered for the treatment of a solid growth of abnormally proliferating cells, comprising administering an antioxidant prior to, with, or following the antineoplastic treatment, as well as a method to increase the therapeutic index of

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an antineoplastic agent administered for the treatment of a solid growth of abnormally proliferating cells, comprising administering an antioxidant prior to, with, or following the antineoplastic treatment.

At least in certain cell lines, it has been discovered that antioxidants increase the cytotoxicity of antineoplastic drugs by affecting a post translational modification of C/EBPβ (CCAAT/Enhancer Binding Protein (C/EBP) β), also known as NF_IL6, AGP/EBP, LAP, IL-6DBP, rNF_IL6, and CRP2(5-11)), which is a member of a diverse group of nuclear transcription factors that contain a leucine zipper motif required for dimer formation and a basic DNA binding domain which facilitates the interactions between these factors and the regulatory domains of promoters and/or enhancers of target genes. C/EBPβ activates several acute-phase protein genes through the NF_IL6 responsive elements, implying that it has a nuclear target(s). C/EBPβ also has been shown to be responsible for the regulation of genes encoding albumin, c-fos, and several adipocyte-specific proteins. Furthermore, C/EBPβ has been implicated in the activation of various genes involved in inflammatory and immune responses, including the interleukin-1 (IL-1) and interleukin-8 (IL-8), granulocyte macrophage/colony-stimulating factor, and immunoglobulin genes. Thus, C/EBPβ is a pleiotropic transactivator involved in a myriad of signal transduction and cell differentiation events.

In one pathway which may not be exclusive, it has been discovered that antioxidants increase the cytotoxicity of antineoplastic drugs through a cascade of events that include: (I) increasing the level of cAMP, which causes the activation of

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protein kinase A, an enzyme which phosphorylates C/EBPβ, which on

phosphorylation is then translocated from the cytosol to the nucleus of the cell wherein

it mediates the induction of p21, which causes an arrest of cell growth; and (ii)

preventing the dephosphorylation of C/EBPβ in the nucleus (and thus deactivation and

delocalization) through the inhibition of protein phosphatase 2A (PP2A). The

inhibition of PP2A activity is caused by a decrease in methyltransferase activity, an

enzyme which carboxymethylates the catalytic subunit of PP2A, which is involved in

maintaining PP2A in an active form. Decreased methylcarboxylation results in

decreased PP2A enzymatic dephosphorylation of C/EBPβ as a substrate. By

simultaneously inducing the phosphorylation of C/EBPβ and inhibiting the

dephosphorylation of C/EBPβ, antioxidants maintain C/EBPβ in an active state in the

nucleus of the cell, which induces the continued expression of a cyclin-dependent

kinase inhibitor, $p21^{WAF1/CIP1}$, and subsequent cell cycle arrest.

Therefore, more generally, the invention includes a method for increasing the

localization of the C/EBPβ protein in the nucleus of a cell that includes the step of

administering an antioxidant to the interior of the cell. It has been discovered that this

method maintains the C/EBPβ protein in an active, phosphorylated state, which

induces cell growth arrest and apoptosis.

As one nonlimiting example, the present invention demonstrates that

compounds exhibiting antioxidant properties (for example, pyrrolidinedithiocarbamate

("PDTC") and the vitamin E analogue, Trolox®) decrease DNA replication in human

colorectal cancer cells by the induction of G_1 cell cycle arrest and/or apoptosis.

However, antioxidant compounds have no effect on normal human colonocytes,

keratinocytes or mammary epithelial cells (see Table 1). Cell cycle perturbations were

more pronounced in colorectal cancer cells expressing mutant p53 compared to wild

type p53. Induction of cell cycle arrest and apoptosis correlated with sustained

5   induction of the cyclin-dependent kinase inhibitor, p21^{WAF1CIP1}. Treatment with

antioxidants in combination with 5-FU significantly reduced anchorage-independent

colorectal cancer cell growth. Furthermore, antioxidants alone significantly reduced

growth of established colorectal cancer tumors in athymic mice, and the combination

of 5-FU and antioxidant either arrested tumor growth (Trolox®) or caused tumor

10   regression (pyrrolidinedithiocarbamate).

DKO-I cells (a human colorectal cancer cell line) constitutively expressing an

epitope-tagged C/EBPß protein were used to further investigate whether a post-

translational modification (phosphorylation) of C/EBPß is responsible for the observed

increase in C/EBPß activity. *In vivo* labeling with [^{32}P]orthophosphate followed by

15   immunoprecipitation revealed a four to six fold increase in the phosphorylation of

epitope tagged C/EBPß in response to PDTC or forskolin (3R-

3α,4aβ,5β,6β.6aα,10α,10aβ,10bα)

-5-(acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-

1H-naphtho[2,1-b]pyran-1-one) with no change in the amount of protein. As a means

20   of mapping the *in vitro* phosphorylation site(s) within C/EBPß, deletion analysis of

C/EBPß was performed. Truncated versions of C/EBPß that contained only the 160 or

200 COOH-terminal amino acids were poor substrates for PDTC-induced

phosphorylation, whereas mutant C/EBPß that contained the 305 COOH-terminal amino acids was phosphorylated by PDTC as efficiently as the full-length C/EBPß. Closer inspection of the primary amino acid sequence between 236 and 305 revealed that this region contained a consensus PKA phosphorylation site (Arg-X-Ser^{299}-X).

It appears that phosphorylation of Ser^{299} of C/EBPß following activation of the cAMP-dependent protein kinase-mediated pathway, is critical for nuclear translocation of this protein and subsequent transactivation of genes in response to an altered intracellular redox status.

The deactivation of C/EBPß in the nucleus occurs by dephosphorylation of this transcriptional factor by protein phosphatase 2A (PP2A). This enzyme is activated by carboxymethylation of the catalytic subunit of PP2A by methyltransferase. In further experiments, it was established that carboxymethylation of the catalytic subunit of protein phosphatase 2A is inhibited by PDTC as an exemplary antioxidant, and further that the loss of carboxymethylation is caused by an inhibition of activity of methyltransferase. These results support the fact that antioxidants prevent the dephosphorylation of C/EBPβ in the nucleus (and thus deactivation) by protein phosphatase 2A (pp2A) by inhibition of methyltransferase which is involved in maintaining protein phosphatase 2A in an active form. Decreased methylcarboxylation results in decreased PP2A enzymatic dephosphorylation of C/EBPβ as a substrate. It was further discovered that the evaluated antioxidants have little or no effect on protein phosphatase 1 (PP1).

The figures presented herein illustrate preferred embodiments of the invention and are not considered to limit the scope of the invention.

Figure 1A is a graph of the concentration of PDTC versus soft-agar forming units x 10^6 HCT 15 and HCT 116 cells (as a percent of control). The graph shows that pyrrolidinedithiocarbamate (PDTC) and vitamin E inhibit anchorage-independent growth *in vitro*. Soft-agar colony formation was measured by seeding HCT 116 or HCT 15 cells in soft agar supplemented with either medium alone (control), or increasing concentrations of pyrrolidinedithiocarbamate (25-200 μM) or vitamin E (0.1-10 mM). Colonies were scored at the end of 10 days incubation at 37°C. Values are representative of three experiments carried out in quadruplicate.

Figure 1B is a series of flow cytometric analyses that indicate that antioxidants induce G_1 cell cycle arrest and apoptosis in CRC cells. Unsynchronized HCT 116 or HCT 15 cells are grown either in the presence or absence of pyrrolidinedithiocarbamate (70 μM) or vitamin E (3 mM). Twenty-four hours following antioxidant exposure, cells were harvested and flow cytometric analysis performed.

Figure 1C is a bar graph of the hours after test compound treatment versus intracellular H_2O_2 levels. Figure 1C illustrates the negative association between intracellular redox status and cell cycle perturbations. Changes in the intracellular redox status were determined by the measurement of endogenous H_2O_2 levels. Background fluorescence was subtracted from each reading. Values are expressed as

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corrected DHR mean per 10^4 cells $\pm$ s.e.m. The percentage of G_1 (circle) or apoptotic

(TUNEL-positive: square) cells by flow cytometric analysis.

Figure 1D shows the effect of N-acetylcysteine, vitamin C and catalase on

endogenous H_2O_2 levels and cell cycle progression. HCT 15 cells were incubated with

pyrrolidinedithiocarbamate (70 μM), vitamin E (3 mM), N-acetylcysteine (50 μM) or

vitamin C (200 μM) for 24 hours. Endogenous H_2O_2 levels and cell cycle changes

were measured as described in Figure 1C. In addition, cells were transiently

transfected with an empty plasmid or an expression plasmid for human catalase and

assayed 24 hours later as above. Values from cells transfected with the empty plasmid

were subtracted from those obtained from the catalase-containing cells and were

expressed as mean $\pm$ s.e.m. from duplicate dishes.

Figure 1E shows that pyrrolidinedithiocarbamate and vitamin E augment 5-FU

or doxorubicin induced growth inhibition *in vitro*. HCT 116 and HCT 15 cells were

seeded in soft agar as described above containing increasing concentrations of either

5-FU (5 X 10^{-8}-5 X 10^{-5} M) or doxorubicin (1 X 10^{-9}-1 X 10^{-6} M), in the presence or

absence of pyrrolidinedithiocarbamate (70 μM) or vitamin E (3 mM). Colonies were

scored after 10 days and IC_{50} values were calculated as the concentration of 5-FU or

doxorubicin required to reduce basal colony formation by 50% ($\pm$ s.e.m.). Values are

representative of three experiments carried out in quadruplicate.

Figure 2A is a graph and associated photographs of tumor-bearing mice which

indicate that pyrrolidinedithiocarbamate and vitamin E enhance 5-FU efficacy in wild

type p53 human CRC tumor xenografts. HCT 116 CRC cells were injected

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subcutaneously between the scapula of *nu/nu* mice. Once tumors reached

approximately 150 mm³, the animals either received weekly i.p. injections of

pyrrolidinedithiocarbamate (70 μM) or vitamin E (3 mM), 5-FU (40 mg/kg) or saline,

or both an antioxidant and 5-FU. Tumor volume was calculated weekly. The

5   photograph shows the effect of indicated treatments on gross tumor size after 4 weeks

of treatment.

. **Figure 2B** is a graph of the weeks of treatment with various test materials

versus tumor volume (mm³) shows that the enhanced efficacy of

pyrrolidinedithiocarbamate and 5-FU as primary treatment and as a salvage regimen

10   for mutant p53 tumors. HCT 15-derived tumors were generated as described above.

Animals were then treated with pyrrolidinedithiocarbamate, in the presence or absence

of 5-FU, for 3 weeks. At this point, treatments were discontinued in animals receiving

the combined treatment of pyrrolidinedithiocarbamate and 5-FU for 2 months. All

other treatment groups received both 5-FU and pyrrolidinedithiocarbamate for the

15   remaining 3 weeks.

Figures 3A-3C show that pyrrolidinedithiocarbamate bypasses p53 to induce

$p21^{WAF1/CIP1}$ expression.

Figure 3A shows that $p21^{WAF1/CIP1}$ protein levels were increased in human CRC

cells expressing functional (HCT 116) and mutant (HCT 15) p53 following

20   pyrrolidinedithiocarbamate treatment. CRC cells were treated as indicted with

pyrrolidinedithiocarbamate (70 μM) and subjected to Western blot analysis.

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Figure 3B shows p53-independent induction of $p21^{WAF1/CIP1}$ mRNA by

pyrrolidinedithiocarbamate in human CRC cells. Exponentially growing,

asynchronous human CRC cells were incubated in serum-containing medium with 70

µM pyrrolidinedithiocarbamate. In addition, HCT 116 cells containing HPV16 E6 to

5   target degradation of p53 were analyzed. Cells were collected at indicated time points

and prepared for poly(A)⁺ mRNA isolation. Samples (3 µg) were electrophoresed

through a 1% (w/v) formaldehyde/agarose gel and transferred onto nitrocellulose

membranes. Northern blotting hybridization was performed at 43°C with a ^{32}P-

labeled $p21^{WAF1/CIP1}$ probe. IB15 is shown as a control for equivalent loading and

10   transfer.

Figure 3C shows that antioxidant-induced apoptosis requires $p21^{WAF1/CIP1}$

expression. HCT 116 cells, containing either functional (p21 +/+) or deleted

$p21^{WAF1/CIP1}$, were treated with the indicated concentrations of

pyrrolidinedithiocarbamate or vitamin E for 24 hours and apoptosis was determined by

15   TUNEL analysis. Values are expressed as percent TUNEL positive cells and

represent mean ± s.e.m. of triplicate measurements.

Figure 4A shows that pyrrolidinedithiocarbamate induces $p21^{WAF1/CIP1}$

transcriptional activity via the NF_IL6 consensus sequence. A 2.4 kilobase pair

$p21^{WAF1/CIP1}$ promoter sequence and mutants were fused to a luciferase reporter gene.

20   TATA represents the $p21^{WAF1/CIP1}$ TATA box located 45 bp from the transcription start

site (defined as +1). -2280, -2198, -2078, -1838, -1428, and 1138 define 5' end points

for terminal deletion constructs. The -2280 ΔNF_IL6 construct contains the intact

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promoter with a two base pair mutation at the NF_IL6 site. All reporter constructs

were transfected into HCT 116 or HCT 15 cells, and antioxidant-induced luciferase

activity was measured in relative light units (RLU) after 24 hours. Luciferase activity

was normalized to CAT activity, and results were reported as fold activation above

5   basal levels.

Figure 4B shows that pyrrolidinedithiocarbamate treatment induces C/EBPβ

DNA binding activity. Left panel: HCT 116 and HCT 15 cells were treated with 70

μM pyrrolidinedithiocarbamate for the indicated times, nuclear extracts were

incubated with a γ-^{32}P-labeled p21-NF_IL6 oligonucleotide. Right panel: Lanes 1-3,

10   competition controls were performed on a nuclear extract derived from HCT 116 cells

treated with pyrrolidinedithiocarbamate for 12 hours (lane 1), with excess unlabeled

wild-type (lane 2) and mutant (lane 3) oligonucleotide. Lanes 4-6, supershift analysis

were performed with C/EBPα (lane 4), β(lane 5), or δ (lane 6) polyclonal antibodies.

Figure 4C and D shows that C/EBPβ can stimulate p21$^{WAF1/CIP1}$ promoter

15   activity. HCT 116 (Fig. C) or HCT 15 (Fig. D) cells were transfected with the

indicated amounts of cytomegalovirus (CMV) expression plasmids, containing

C/EBPα, β or δ cDNAs, and 3 μg of p21$^{WAF1/CIP1}$-luciferase. A control plasmid was

included in Figure 4A.

Figure 4E shows that C/EBPβ regulates cellular sensitivity to antioxidant-

20   induced apoptosis. Control HCT 15 cells and sense or antisense C/EBPβ cell lines

were grown in the presence or absence of 10 μ M muristerone A and/or

pyrrolidinedithiocarbamate (70 μM) or vitamin E (3 mM) for 24 hours. The apoptotic

indices were estimated by the percentage of TUNEL-positive cells scored under a light

microscope at 200-fold magnification and values are expressed as mean ± s.e.m. for

triplicate samples. The inset shows a representative Western blot for p21$^{WAF1/CIP1}$

protein levels in both transfected cell lines, grown in the presence or absence of 10 μM

5   muristerone A.

Figure 4F shows that elevated C/EBPβ protein levels enhance

chemotherapeutic agent cytotoxicity *in vitro*. Control HCT 15 cells and the sense

C/EBPβ cell line were induced with 10 μM muristerone A and exposed to either 5-FU

(1.5 μM) or doxorubicin (0.1 μM) for 24 hours. The apoptotic index was calculated as

10   described in Figure 4C.

Figures 5a and 5b are bar graphs of the growth of BrDU-labelled cells

(percent of total cell nuclei; BrDU refers to bromodeoxyuridine) from colorectal cell

xenografts derived from athymic mice treated with saline, vitamin E, PDTC, 5-FU,

and the combination of vitamin E and 5-FU, as a measure of the effect of the test

15   compound on proliferation of HCT 116 and HCT 15 cells.

Figures 6a and 6b are bar chart graphs of TUNEL-positive cells from

colorectal cell xenografts derived from athymic mice (percent of total cell nuclei;

TUNEL REFERS TO TdT-mediated dUTP-nick-end-labeling) treated with saline,

vitamin E, PDTC, 5-FU, and the combination of vitamin E and 5-FU, as a measure of

20   the effect of the test compound on apoptosis. Tumor tissues were fixed overnight in

4% (v/v) paraformaldehyde and embedded in paraffin according to standard

histological procedures. Sections were pretreated with 10 mM citrate buffer (pH 6.0)

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and incubated with PC10 monoclonal antibody against BrDU (Boehringer

Mannheim). TdT labeling of fragmented DNA (TUNEL) was performed according to

manufacturer's instructions. The proliferative index (percent of total BrDU cell

nuclei) and the apoptotic index (TUNEL) were estimated by the percentage of cells

5   scored under a microscope at 200-fold magnification.

Figures 7A-7D illustrate that PDTC treatment induces C/EBPβ DNA binding

activity via a post-translational modification. (A) DKO-1 cell were treated with 70μM

PDTC for the indicated times, nuclear extracts were prepared with a [γ-^{32}P]-labeled

p21-NF_IL6 oligonucleotide (Lanes 1-9). Specificity assays: Lanes 10-12,

10   competition controls were performed on a nuclear extract derived from DKO-1 cells

treated with PDTC for 3 hours (lane 5), with excess unlabeled wild-type (lane 11) and

mutant (lane 12) oligonucleotide. Lanes 13-15, supershift analyses were performed

with C/EBPα (lane 13), β (lane 14), or δ (lane 15) polyclonal antibodies. (B) Parallel

DKO-1 cell cultures were treated with PDTC (70 μM) for the indicated times.

15   Poly(A) was isolated and treatment-related variations in C/EBPβ mRNA levels were

evaluated by Northern blot analysis. IB15 is shown as a control for equivalent loading

and transfer. (C) Parallel DKO-1 cultures were treated with PDTC (70μM) in the

presence of [^{32}P]orthophosphate. C/EBPβ from cytosolic and nuclear fractions were

purified by immunoprecipitation from cells before (time 0) or at the indicated times

20   after PDTC treatment. Treatment-related variations in the localization of C/EBPβ

were analysed by SDS-PAGE followed by autoradiography or Western blot analysis

(100 μg of total cellular protein / lane). (D) DKO-1 cells were cultured in the

presence of PDTC (70 μM) for 1 hour and then processed for immunocytochemistry to detect treatment-related differences in the compartmentalization of C/EBPβ protein. In all experiments, parallel cultures treated with preimmune sera or primary anti-C/EBPβ antisera that had been preincubated with *in vitro* translated C/EBPβ protein demonstrated no fluorescent signal after treatment with the secondary Cy3-conjugated antibody. Representative photomicrographs show anti-C/EBPβ stained cells before and after PDTC treatment.

Figures 8A-8B illustrate the effect of PDTC on endogenous cAMP levels and PKA activity. DKO-1 cells were treated with 70µM PDTC for the indicated times. Cell lysates were prepared and assayed for (A) endogenous cAMP levels or (B) PKA activity. The values are expressed as pmol mean per µg protein ± s.e.m. and are representative of three experiments carried out in quadruplicate.

Figures 9A-9C illustrate that PDTC phosphorylates C/EBPβ at Ser[299]. (A) Endogenous C/EBPβ from [^{32}P]orthophosphate-labeled DKO-1 cells (2 mCi/ml. 3 h) that were treated with either 0 µM (lane 1), 70 µM PDTC (lane 2) or 50 µM forskolin were immunoprecipitated with anti C/EBPβ antibodies. Labeled proteins were visualized by SDS-PAGE followed by autoradiography. (B) Tryptic phosphopeptide maps of *in vivo* labeled epitope-tagged C/EBPβ. Wild type (WT) and mutant (Ala[299]) C/EBPβ, immunoprecipitated from PDTC treated or untreated DKO-1 cells with the antibody to the FLAG-epitope, were digested with trypsin and the phosphopeptides separated by electrophoresis and thin-layer chromatography and visualized by autoradiography, $X_{1,2}$ were constitutively phosphorylated. The level of

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phosphopeptide X_3 was increased after PDTC treatment in cells transfected with the wild type, but not mutant, protein. The circle indicates the origin. (C) Comparison of the *in vivo* phosphorylation of wild type and Ala substitution mutants of C/EBPβ from untreated cells and cells treated with PDTC. Autoradiography (top) and C/EBPβ immunoblot (bottom) are shown.

Figures 10A-10B illustrate that PKA phosphorylation of C/EBPβ is required for nuclear translocation. (A) Parallel DKO-1 cell cultures were treated with PDTC (0 or 70 μM) for 3 hours. Poly(A)$^+$ mRNA and protein were isolated from each group and treatment-related variations in C/EBPβ mRNA and protein levels were evaluated by Northern or Western blot analysis. IB15 is shown as a control for equivalent loading and transfer. (B) DKO-1 cells were treated with PDTC (0 or 70μM) or PDTC and mPKI (myristylated protein kinase A inhibitor; 1 μM) for 3 hours. Cells were fixed with paraformaldehyde and C/EBPβ protein visualized by immunofluorescence staining. Treatment of cells with mPKI alone failed to induce nuclear translocation of C/EBPβ (data not shown).

Figure 11 illustrates that carboxymethylation of the catalytic subunit of protein phosphatase 2A is inhibited by PDTC. DKO-1 cells were incubated in serum-containing media containing [methyl-^{3}H]S-adenosyl methionine and/or 70 μM PDTC for three hours. Cytosolic or nuclear fractions were prepared and C/EBPβ immunoprecipitated using standard methods. Antibody/antigen complexes were resolved by SDS-PAGE and the presence of PP2Ac was detected by fluorography.

PDTC inhibited carboxymethylation of PP2A subunit in nuclear fractions and to a lesser extent, in cytosolic fractions.

Figure 12 illustrates that PDTC inhibits methyltransferase activation of PP2Ac. PP2A (a and c dimer) was incubated in the presence of [methyl-^{3}H]S-adenosyl methionine, increasing concentrations of PDTC and partially purified rat methyltransferase for thirty minutes at 37 degrees C. The reaction was terminated by the addition of SDS-sample buffer. Samples were resolved by SDS-PAGE and the presence of methylated PP2A catalytic subunit visualized by fluorography. As indicated, PDTC selectively inhibits the ability of methyltransferase to carboxymethylate the catalytic subunit of PP2A in a dose dependent manner.

Figure 13 is a graph of time in hours versus percent radioactivity remaining on the protein substrate. The figure indicates that PDTC inhibits PP2A, but not PP1, activity. The activity of PDTC is compared to I2 (a selective PP1 inhibitor), okadaic acid (an inhibitor of both PP2A and PP1), I2 and PDTC, and okadaic acid and PDTC. DKO-1 cells were grown in the presence of PDTC (test) or not (control). The cells were lysed, and then radioactive phosphorylated C/EBPβ was added. The test compounds were then added, and incubated with the lysate. The protein was collected and the amount of radioactive phosphate remaining in the protein measured.

Figure 14 illustrates that the transcription factor C/EBPβ is complexed with the PP2Ac protein phosphatase. Rat brain soluble extracts were fractionated by phenyl-Sepharose and analyzed for methyltransferase activity using exogenous PP2A heterodimer (a-c complex). The peak of methyltransferase activity was further

fractionated by Source Q, a strong anion exchange, and gel filtration chromatography. The partially purified methyltransferase illustrated in Figure 14 represents the peak methyltransferase activity from the gel filtration column. This peak fraction of methyltransferase activity is taken further to DEAE, a weak anion exchange, and

5   MonoQ, a different strong anion exchange resin, columns. Both C/EBPb and PP2A are detectable following these additional steps. Rat brain extracts are shown as a positive control (C/EBPβ and PP2Ac migrate at approximately 45 and 36 kDa on SDS-PAGE).

DETAILED DESCRIPTION OF THE INVENTION

10   It has been discovered that antioxidants induce cell cycle arrest and apoptosis in abnormally proliferating cells through a mechanism mediated by the activation of the transcription factor C/EBPβ that binds to a specific site in the p21 promoter to induce p21 expression independent of p53. It has also been discovered that a site-selective phosphorylation at Ser^{299} of C/EBPβ by protein kinase A following antioxidant

15   treatment is essential for the observed nuclear translocation of this protein.

It has also been discovered that antioxidants prevent the dephosphorylation of C/EBPβ in the nucleus (and thus deactivation and delocalization) through the inhibition of PP2A. The inhibition of PP2A activity is caused by a decrease in methyltransferase activity, an enzyme which carboxymethylates the catalytic subunit

20   of PP2A, which is involved in maintaining PP2A in an active form. Decreased methylcarboxylation results in decreased PP2A enzymatic dephosphorylation of C/EBPβ as a substrate. By simultaneously inducing the phosphorylation of C/EBPβ

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and inhibiting the dephosphorylation of C/EBPβ, antioxidants maintain C/EBPβ in an active state in the nucleus of the cell, which induces the continued expression of p21$^{WAF1/CIP1}$ and subsequent cell cycle arrest.

The methyltransferase responsible for PP2A subunit carboxymethylation *in vivo* and *in vitro* represents a unique type of carboxyl methyltransferase. The mammalian type II and type III carboxyl methyltransferases appear to have substantially different properties from the enzyme that carboxymethylates the PP2Ac subunit. Protein carboxyl methyltransferase type II modifies D-aspartyl and L-isoaspartyl residues that accumulate in proteins with aging, and, therefore methylates a different protein containing these amino acids. Carboxyl methyltransferase type III modifies proteins at cysteine, proceeding to the carboxyl terminus of proteins (G-proteins), and requires isoprenylation of cysteine and proteolytic cleavage of the last three carboxyl-terminal residues. Activity of this carboxyl methyltransferase is not altered by antioxidant treatment of colorectal cancer cell line DKO-1 *in vitro*. Therefore, *in vitro* data suggests that antioxidants selectively inhibit the methyltransferase responsible for PP2Ac, but not G-protein, methylation.

A novel higher order protein complex has also been identified that consists of C/EBPβ, PP2A, and methyltransferase. Thus, another embodiment of this invention is this novel complex in isolated form, for example in at least 70%, and preferably 80 or 90% purity. A method for isolating this enzyme is provided in Example 27.

I. Embodiments of the Invention

Based on the fundamental discoveries described herein, a method for increasing the localization of the C/EBPβ protein in the nucleus of a cell is presented that includes the step of administering an antioxidant to the interior of the cell. It has been

5   discovered that this method maintains the C/EBPβ protein in an active, phosphorylated state, which induces cell growth arrest and apoptosis.

In one embodiment, the invention is a method to enhance the cytotoxic activity of an antineoplastic drug comprising administering an effective amount of the antineoplastic drug to a host in need of treatment in combination with an effective

10   cytotoxicity-increasing amount of an antioxidant. It has been discovered that antioxidants, including those specifically disclosed herein, induce cell cycle arrest (G1, G2, S and M type), and thus are useful to enhance the efficacy of antineoplastic drugs for the treatment of disorders associated with abnormal cell proliferation. It has been discovered that this method maintains the C/EBPβ protein in an active,

15   phosphorylated state, which induces cell growth arrest and apoptosis. In an alternative embodiment, a method is presented to increase the cytotoxicity of an antineoplastic or chemotherapeutic agent against a disorder of abnormal cell hyperproliferation, that includes increasing the phosphorylation state of C/EBPβ protein in a host, for example, an individual or animal in need of such treatment, comprising the step of

20   administering to said individual or animal a cytotoxicity-increasing dose of an antioxidant in combination or alternation with a pharmacologically effective dose of a chemotherapeutic agent.

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In another embodiment, the invention is directed to a method of treating a host having a neoplastic condition, comprising the step of administering to the host a therapeutically effective dose of a cytotoxic chemotherapeutic therapy and an antioxidant, wherein the cytotoxic chemotherapeutic therapy is selected from the group consisting of cancer chemotherapeutic agents and radiation therapy. Representative cancer chemotherapeutic agents and antioxidants are listed below. Any radiation therapy that ameliorates a condition of abnormal cellular proliferation is appropriate for use in this method, including ionizing radiation that is particulate or electromagnetic. Suitable and effective dosages of radiation therapy for a wide variety of neoplastic conditions are well known. In one nonlimiting embodiment, radiation therapy is gamma irradiation given at a dose of from about 3,000 centigrey to about 5,000 centigrey over an appropriate time frame, for example, up to six weeks.

The present invention is also directed to a method of increasing expression of the p21 protein as a means to arrest cell growth and induce apoptosis in an individual in need of such treatment, comprising the step of administering to said individual a pharmacologically effective dose of an antioxidant, or a combination of an antioxidant and an antineoplastic agent.

The present invention is further directed to a method of regulating cell cycle arrest (G_1, G_2, S or M) and apoptosis in an individual in need of such treatment, comprising the step of administering to said individual a pharmacologically effective dose of an antioxidant or a combination of an antioxidant and a antineoplastic agent.

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In another embodiment, therapeutic efficacy may be achieved by administration of an effective amount of C/EBPβ, or a protein with substantial homology to C/EBPβ, to achieve the effects described in detail herein. The protein or protein analog can be administered alone or as an adjunct to antineoplastic therapy. A protein with substantial homology to C/EBPβ is defined herein as consisting of or containing a peptide sequence of the form -X1-Arg-X2-Ser-X3 wherein X2 is the C/EBPβ amino acid at position 298, and X1 and X3 represent flanking peptide sequences with substantial homology to those of C/EBPβ. The term substantial homology refers to a protein or peptide sequence that performs substantially the same function as the parent sequence and has at least 60%, or more preferably, 75%, and most preferably, 90% or 95% or greater, sequence identity. Methods for the effective delivery of proteins are known and can be employed in conjunction with this embodiment to enhance the efficacy of this therapy.

In another embodiment, a synthetic Ser299 phosphorylated C/EBPβ analog can be administered that has a stabilized phosphate bond that is resistant to dephosphorylation. Such stabilized phosphates include, but are not limted to, phosphoroamidates and phosphonate analogs.

The invention also provides a method of inhibiting protein phosphatase 2A (PP2A) in a cell that includes administering to the interior of the cell a protein phosphatase-inhibiting amount of an antioxidant. In an alternative embodiment of this aspect of the invention, a method of decreasing the carboxymethylation status of the

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catalytic subunit of PP2A is provided that includes contacting a cell with a methyltransferase- or methylesterase-inhibiting amount of an antioxidant.

In one pathway which may not be exclusive, it has been discovered that antioxidants increase the cytotoxicity of antineoplastic drugs through a cascade of events that include: (i) increasing the level of cAMP, which causes the activation of protein kinase A, an enzyme which phosphorylates C/EBPβ, which on phosphorylation is then translocated from the cytosol to the nucleus of the cell wherein it mediates the induction of p21, which causes an arrest of cell growth; and (ii) preventing the dephosphorylation of C/EBPβ in the nucleus (and thus deactivation and delocalization) through the inhibition of PP2A. The inhibition of PP2A activity is caused by a decrease in methyltransferase activity, an enzyme which carboxymethylates the catalytic subunit of PP2A, which is involved in maintaining PP2A in an active form. Decreased methylcarboxylation results in decreased PP2A enzymatic dephosphorylation of C/EBPβ as a substrate. By simultaneously inducing the phosphorylation of C/EBPβ and inhibiting the dephosphorylation of C/EBPβ, antioxidants maintain C/EBPβ in an active state in the nucleus of the cell, which induces the continued expression of p21$^{WAF1/CIP1}$ and subsequent cell cycle arrest.

Based on this discovery, a method for the identification of therapeutically effective compounds for the treatment of abnormal cell proliferation is presented that includes assessing the compound's ability to increase the localization of the C/EBPβ protein in the nucleus of a cell. In an alternative embodiment, a method for the identification of therapeutically effective compounds for the treatment of abnormal

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cell proliferation is presented that includes assessing the compound's ability to increase the phosphorylation at Ser299 of C/EBPβ. This method includes incubating a selected cell line with a test compound for a predetermined time (for example three hours) at 37 degrees C followed by immunoprecipitation of C/EBPβ from the nuclear

5   fraction. Tryptic digestion and thin layer chromatography is then carried out to confirm phosphorylation of C/EBPβ.

Based on the discoveries described in detail herein, one of ordinary skill will understand that the invention further includes, but is not limited to the following aspects.

10   (i) A method for the identification of therapeutically effective compounds by assessing the ability of the compound to alter the phosphorylation status of C/EBPβ at Ser299. In this method, the test compound is included in a solution that contains at least phosphorylated C/EBPβ, a dimeric form of protein phosphatase 2A containing a and c subunits, methyltransferase and [methyl-3H]S-

15   adenosyl methionine.

(ii) A method for the identification of therapeutically effective compounds by assessing the ability of the compound to inhibit protein phosphatase 2a activity, using the method described in (1) or another protocol known or obvious to those skilled in the art.

20   (iii) A method for the identification of therapeutically effective compounds by assessing the ability of the compound to alter the carboxymethylation status of protein phosphatase 2a.

(iv) A method for the identification of therapeutically effective compounds by assessing the ability of the compound to alter the activity of methyltransferase.

(v) A peptide sequence of the form -X1-Arg-X2-Ser-X3 wherein X2 is the C/EBP-β amino acid at position 298, and X1 and X3 represent flanking peptide sequences with substantial homology to C/EBPβ.

(vi) A method for the enhancement the phosphorylation status and functionality of C/EBPβ induced by mediators including, but not limited to, cAMP dependent protein kinases, protein kinase C, ras-dependent MAP kinase and calcium-calmodulin dependent kinase, in an individual or animal in need of such treatment comprising the step of administering to said individual or animal a pharmacologically effective dose of an antioxidant that increases the nuclear residence time and functionality of C/EPB-β.

(vii) A method for the enhancement of the phosphorylation status and functionality of C/EBP-β induced by, but not limited to, cAMP dependent protein kinases, protein kinase C, ras-dependent MAP kinase and calcium-calmodulin dependent kinase, in an individual or animal in need of such treatment comprising the step of administering to said individual or animal a pharmacologically effective dose of an antioxidant.

(viii) A method for the treatment of a host, for example, an individual or animal, at risk for developing or exhibiting a neoplastic condition comprising the step of administering to said individual or animal a pharmacologically effective dose of an

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antioxidant.

(ix) A method for the the treatment of individuals or animals at risk for developing a neoplastic condition that includes increasing the nuclear localization of C/EBPβ expression and function.

5   (x) A method for the treatment of individuals with a disorder of abnormal cell proliferation, including but not limited to benign and malignant tumors, that includes the step of administering to said individual or animal a pharmacologically effective dose of a therapeutic that increases the nuclear residence time of C/EPBβ, and wherein the therapeutic is either an antioxidant alone or a combination of an antioxidant and

10   antineoplastic agent.

(xi) A method for the diagnosis and assessment of response to treatment of individuals with neoplastic and cell proliferative diseases through the measurement either alone or in combination, of C/EBPβ activation, phosphorylation and nuclear residence time of C/EBPβ, PP2A inhibition of carboxymethylation of the catalytic

15   subunit of PP2A, and inhibition of methyltransferase or methylesterase activity.

II. ANTIOXIDANTS

As used herein, the term antioxidant refers to a substance that prevents the oxidation of an oxidizable compound under physiological conditions. In one

20   embodiment, a compound is considered an antioxidant for purposes of this disclosure if it reduces endogenous oxygen radicals in vitro. The antioxidant can be added to a cell extract under oxygenated conditions and the effect on an oxidizable compound

evaluated. As nonlimiting examples, antioxidants scavenge oxygen, superoxide anions, hydrogen peroxide, superoxide radicals, lipooxide radicals, hydroxyl radicals, or bind to reactive metals to prevent oxidation damage to lipids, proteins, nucleic acids, etc. The term antioxidant includes, but is not limited to, the following classes of

5 compounds.

A) Dithiocarbamates

Dithiocarbamates have been extensively described in patents and in scientific literature. Dithiocarbamates and related compounds have been reviewed extensively for example, by G. D. Thorn et al entitled "The Dithiocarbamates and Related

10 Compounds," Elsevier, New York, 1962. U.S. Patent Nos. 5,035,878 and 5,294,430 disclose that dithiocarbamates can reverse the damage to the blood-forming function of the bone marrow (myelosuppression) caused by treatment with antineoplastic agents. All of the pharmaceutically acceptable dithiocarbamates disclosed in these two patents which increase the nuclear localization of C/EBPβ are suitable for use in

15 this invention, and are incorporated herein by reference.

Active Compounds

Dithiocarbamates are transition metal chelators clinically used for heavy metal intoxication. Baselt, R.C., F.W.J. Sunderman, et al. (1977), "Comparisons of antidotal

20 efficacy of sodium diethyldithiocarbamate, D-penicillamine and triethylenetetramine upon acute toxicity of nickel carbonyl in rats." Res Commun Chem Pathol Pharmacol 18(4): 677-88; Menne, T. and K. Kaaber (1978), "Treatment of pompholyx due to

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nickel allergy with chelating agents." Contact Dermatitis 4(5): 289-90; Sunderman,

F.W. (1978), "Clinical response to therapeutic agents in poisoning from mercury

vapor" Ann Clin Lab Sci 8(4): 259-69; Sunderman, F.W. (1979), "Efficacy of

sodium diethyldithiocarbamate (dithiocarb) in acute nickel carbonyl poisoning." Ann

5   Clin Lab Sci 9(1): 1-10; Gale, G.R., A.B. Smith, et al. (1981),

"Diethyldithiocarbamate in treatment of acute cadmium poisoning." Ann Clin Lab Sci

11(6): 476-83; Jones, M.M. and M.G. Cherian (1990), "The search for chelate

antagonists for chronic cadmium intoxication." Toxicology 62(1): 1-25; Jones, S.G.,

M.A. Basinger, et al. (1982), "A comparison of diethyldithiocarbamate and EDTA as

10   antidotes for acute cadmium intoxication." Res Commun Chem Pathol Pharmacol

38(2): 271-8; Pages, A., J.S. Casas, et al. (1985), "Dithiocarbamates in heavy metal

poisoning: complexes of N,N-di(1-hydroxyethyl)dithiocarbamate with Zn(II), Cd(II),

Hg(II), CH3Hg(II), and C6H5Hg(II).: J. Inorg Biochem 25(1): 35-42; Tandon, S.K.,

N.S. Hashmi, et al. (1990), "The lead-chelating effects of substituted

15   dithiocarbamates." Biomed Environ Sci 3(3): 299-305.

Dithiocarbamates have also been used adjunctively in cis-platinum

chemotherapy to prevent renal toxicity. Hacker, M.P., W.B. Ershler, et al. (1982).

"Effect of disulfiram (tetraethylthiuram disulfide) and diethyldithiocarbamate on the

bladder toxicity and antitumor activity of cyclophosphamide in mice." Cancer Res

20   42(11): 4490-4. Bodenner, 1986 #733; Saran, M. and Bors, W. (1990). "Radical

reactions in vivo--an overview." Radiat. Environ. Biophys. 29(4):249-62.

A dithiocarbamate currently used in the treatment of alcohol abuse is

disulfiram, a dimer of diethyldithiocarbamate. Disulfuram inhibits hepatic aldehyde

dehydrogenase. Inoue, K., and Fukunaga, et al., (1982). "Effect of disulfiram and its

reduced metabolite, diethyldithiocarbamate on aldehyde dehydrogenase of human

5   erythrocytes." Life Sci 30(5): 419-24.

It has been reported that dithocarbamates inhibit HIV virus replication, and also

enhance the maturation of specific T cell subpopulations. This has led to clinical trials

of diethyldithiocarbamate in AIDs patient populations. Reisinger, E., et al., (1990).

"Inhibition of HIV progression by dithiocarb." Lancet 335: 679.

10   Dithiocarboxylates are compounds of the structure A-SC(S)-B, which are

members of the general class of compounds known as thiol antioxidants, and are

alternatively referred to as carbodithiols or carbodithiolates. It appears that the -

SC(S)- moiety is essential for therapeutic activity, and that A and B can be any group

that does not adversely affect the efficacy or toxicity of the compound.

15   In an alternative embodiment, one or both of the sulfur atoms in the

dithiocarbamate is replaced with a selenium atom. The substitution of sulfur for

selenium may decrease the toxicity of the molecule in certain cases, and may thus be

better tolerated by the patient.

A and B can be selected by one of ordinary skill in the art to impart desired

20   characteristics to the compound, including size, charge, toxicity, and degree of

stability, (including stability in an acidic environment such as the stomach, or basic

environment such as the intestinal tract). The selection of A and B will also have an

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important effect on the tissue-distribution and pharmacokinetics of the compound. The compounds are preferably eliminated by renal excretion.

An advantage in administering a dithiocarboxylate pharmaceutically is that it does not appear to be cleaved enzymatically *in vivo* by thioesterases, and thus may exhibit a prolonged halflife *in vivo*.

In a preferred embodiment, A is hydrogen or a pharmaceutically acceptable cation, including but not limited to sodium, potassium, calcium, magnesium, aluminum, zinc, bismuth, barium, copper, cobalt, nickel, or cadmium; a salt-forming organic acid, typically a carboxylic acid, including but not limited to acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, naphthalenedisulfonic acid, or polygalacturonic acid; or a cation formed from ammonia or other nitrogenous base, including but not limited to a nitrogenous heterocycle, or a moiety of the formula $NR^4R^5R^6R^7$, wherein R^4, R^5, R^6, and R^7 are independently hydrogen, C_{1-6} linear, branched, or (in the case of C_{4-6}) cyclic alkyl, hydroxy(C_{1-6})alkyl (wherein one or more hydroxyl groups are located on any of the carbon atoms), or aryl, N,N-dibenzylethylene-diamine, D-glucosamine, choline, tetraethylammonium, or ethylenediamine.

In another embodiment, A can be a physiologically cleavable leaving group that can be cleaved *in vivo* from the molecule to which it is attached, and includes but is not limited to acyl (including acetyl, propionyl, and butyryl), alkyl, phosphate, sulfate or sulfonate.

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In one embodiment, B is alkyl, alkenyl, alkynyl, alkaryl, aralkyl, haloalkyl, haloalkenyl, haloalkynyl, aryl, alkaryl, hydrogen, C_{1-6} alkoxy-C_{1-10} alkyl, C_{1-6} alkylthio-C_{1-10} alkyl, NR^2R^3, -$(CHOH)_nCH_2OH$, wherein n is 0, 1, 2, 3, 4, 5, or 6, -$(CH_2)_nCO_2R^1$, including alkylacetyl, alkylpropionyl, and alkylbutyryl, or hydroxy(C_{1-6})alkyl- (wherein one or more hydroxyl groups are located on any of the carbon atoms).

In another embodiment, B is NR^2R^3, wherein R^2 and R^3 are independently alkyl;

-$(CHOH)_n(CH_2)_nOH$, wherein n is 0, 1, 2, 3, 4, 5, or 6; -$(CH_2)_nCO_2R^1$, -$(CH_2)_nCO_2R^4$; hydroxy(C_{1-6})alkyl-; alkenyl (including but not limited to vinyl, allyl, and $CH_3CH=CH-CH_2CH_2$); alkyl(CO_2H), alkenyl(CO_2H), alkynyl(CO_2H), or aryl, wherein the aryl group can be substituted as described above, notably, for example, with a NO_2, CH_3, t-butyl, CO_2H, halo, or p-OH group; or R^2 and R^3 can together constitute a bridge such as -$(CH_2)_m$-, wherein m is 3, 4, 5, 6, 7, 8, 9, or 10, and wherein R^4 is alkyl, aryl, alkaryl, or aralkyl, including acetyl, propionyl, and butyryl.

In yet another embodiment, B can be a heterocyclic or alkylheterocyclic group. The heterocycle can be optionally partially or totally hydrogenated. Nonlimiting examples are those listed above, including phenazine, phenothiazine, pyridine and dihydropyridine.

In still another embodiment, B is the residue of a pharmaceutically-active compound or drug. The term drug, as used herein, refers to any substance used internally or externally as a medicine for the treatment, cure, or prevention of a disease

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or disorder. The -C(S)SA group can be directly attached to the drug, or attached through any suitable linking moiety.

In another embodiment, the dithiocarbamate is an amino acid derivative of the structure $AO_2C-R^9-NR^{10}-C(S)SA$, wherein R_9 is a divalent B moiety, a linking moiety, or the internal residue of any of the naturally occurring amino acids (for example, CH_3CH for alanine, CH_2 for glycine, $CH(CH_2)_4NH_2$ for lysine, etc.), and R^{10} is hydrogen or lower alkyl.

B can also be a polymer to which one or more dithiocarbamate groups are attached, either directly, or through any suitable linking moiety. The dithiocarbamate is preferably released from the polymer under *in vivo* conditions over a suitable time period to provide a therapeutic benefit. In a preferred embodiment, the polymer itself is also degradable *in vivo*. The term biodegradable or bioerodible, as used herein, refers to a polymer that dissolves or degrades within a period that is acceptable in the desired application (usually *in vivo* therapy), usually less than five years, and preferably less than one year, on exposure to a physiological solution of pH 6-8 having a temperature of between 25 and 37°C. In a preferred embodiment, the polymer degrades in a period of between 1 hour and several weeks, according to the application.

A number of degradable polymers are known. Nonlimiting examples are peptides, proteins, nucleoproteins, lipoproteins, glycoproteins, synthetic and natural polypeptides and polyamino acids, including but not limited to polymers and copolymers of lysine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic

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acid, glutamine, hydroxylysine, serine, threonine, and tyrosine; polyorthoesters,

including poly(a-hydroxy acids), for example, polylactic acid, polyglycolic acid,

poly(lactide-co-glycolide), polyanhydrides, albumin or collagen, a polysaccharide

containing sugar units such as lactose, and polycaprolactone. The polymer can be a

5   random or block copolymer.

B can also be a group that enhances the water solubility of the dithiocarbamate,

for example, -lower alkyl-O-R^8, wherein R^8 is -$PO_2(OH)^- M^+$ or $PO_3(M^+)_2$ wherein M^+

is a pharmaceutically acceptable cation; -$C(O)(CH_2)_2CO_2^-$ M^+, or -$SO_3^- M^+$; -lower

alkylcarbonyl-lower alkyl; -carboxy lower alkyl; -lower alkylamino-lower alkyl; N,N-

10   di-substituted amino lower alkyl-, wherein the substituents each independently

represent lower alkyl; pyridyl-lower alkyl-; imidazolyl-lower alkyl-; imidazolyl-Y-

lower alkyl wherein Y is thio or amino; morpholinyl-lower alkyl; pyrrolidinyl-lower

alkyl; thiazolinyl-lower alkyl-; piperidinyl-lower alkyl; morpholinyl-lower

hydroxyalkyl; N-pyrryl; piperazinyl-lower alkyl; N-substituted piperazinyl-lower

15   alkyl, wherein the substituent is lower alkyl; triazolyl-lower alkyl; tetrazolyl-lower

alkyl; tetrazolylamino-lower alkyl; or thiazolyl-lower alkyl.

In an alternative embodiment, a dimer such as B-C(S)S-SC(S)-B can be

administered.

The term "alkyl," as used herein, unless otherwise specified, refers to a

20   saturated straight, branched, or cyclic (in the case of C_5 or greater) hydrocarbon of C_1

to C_{10} (or lower alkyl, i.e., C_1 to C_5), which specifically includes methyl, ethyl, propyl,

isopropyl, butyl, isobutyl, t-butyl, pentyl, cyclopentyl, isopentyl, neopentyl, hexyl,

isohexyl, cyclohexyl, cyclohexylmethyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl. The alkyl group can be optionally substituted on any of the carbons with one or more moieties selected from the group consisting of hydroxyl, amino, or mono- or disubstituted amino, wherein the substituent group is independently alkyl,

5   aryl, alkaryl or aralkyl; aryl, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, et al., "Protective Groups in Organic Synthesis," John Wiley and Sons, Second Edition, 1991.

10   The term "alkenyl," as referred to herein, and unless otherwise specified, refers to a straight, branched, or cyclic hydrocarbon of C_2 to C_{10} with at least one double bond.

The term "alkynyl," as referred to herein, and unless otherwise specified, refers to a C_2 to C_{10} straight or branched hydrocarbon with at least one triple bond.

15   The term "aralkyl" refers to an aryl group with at least one alkyl substituent.

The term "alkaryl" refers to an alkyl group that has at least one aryl substituent.

The term "halo (alkyl, alkenyl, or alkynyl)" refers to an alkyl, alkenyl, or alkynyl group in which at least one of the hydrogens in the group has been replaced with a halogen atom.

20   The term "aryl," as used herein, and unless otherwise specified, refers to phenyl, biphenyl, or napthyl, and preferably phenyl. The aryl group can be optionally substituted with one or more moieties selected from the group consisting of alkyl,

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hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid,

sulfate, phosphonic acid, phosphate, or phosphonate, CO_2H, or its pharmaceutically

acceptable salt, CO_2(alkyl, aryl, alkaryl or aralkyl), or glucamine, either unprotected,

or protected as neccesary, as known to those skilled in the art, for example, as taught

5   in Greene, et al., "Protective Groups in Organic Synthesis," John Wiley and Sons,

Second Edition, 1991.

The term "alkoxy," as used herein, and unless otherwise specified, refers to a

moiety of the structure -O-alkyl.

The term "acyl", as used herein, refers to a group of the formula C(O)R',

10   wherein R' is an alkyl, aryl, alkaryl or aralkyl group.

The term "heterocyclic" as used herein, refers to an aromatic moiety that

includes at least one sulfur, oxygen, or nitrogen in the aromatic ring. Nonlimiting

examples are phenazine, phenothiazine, furyl, pyridyl, pyrimidyl, thienyl, isothiazolyl,

imidazolyl, tetrazolyl, pyrazinyl, benzofuranyl, benzothiophenyl, quinolyl,

15   isoquinolyl, benzothienyl, isobenzofuryl, pyrazolyl, indolyl, isoindolyl,

benzimidazolyl, purinyl, morpholinyl, carbozolyl, oxazolyl, thiazolyl, isothiazolyl,

1,2,4-thiadiazolyl, isooxazolyl, pyrrolyl, pyrazolyl, quinazolinyl, pyridazinyl,

pyrazinyl, cinnolinyl, phthalazinyl, quinoxalinyl, xanthinyl, hypoxanthinyl, pteridinyl,

5-azacytidinyl, 5-azauracilyl, triazolopyridinyl, imidazolopyridinyl,

20   pyrrolopyrimidinyl, pyrazolopyrimidinyl, adenine, N^6-alkylpurines, N^6-benzylpurine,

N^6-halopurine, N^6-vinylpurine, N^6- acetylenic purine, N^6-acyl purine, N^6-

hydroxyalkyl purine, N^6-thioalkyl purine, thymine, cytosine, 6-azapyrimidine, 2-

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mercaptopyrmidine, uracil, N^5-alkylpyrimidines, N^5-benzylpyrimidines, N^5-halopyrimidines, N^5-vinylpyrimidine, N^5-acetylenic pyrimidine, N^5-acyl pyrimidine, N^5-hydroxyalkyl purine, and N^6-thioalkyl purine, and isoxazolyl. The heterocyclic group can be optionally substituted as described above for aryl. The heterocyclic

5   group can be partially or totally hydrogenated as desired. As a nonlimiting example, dihydropyridine can be used in place of pyridine. Functional oxygen and nitrogen groups on the heterocyclic base can be protected as necessary or desired during the reaction sequence. Suitable protecting groups are well known to those skilled in the art, and include trimethylsilyl, dimethylhexylsilyl, t-butyldimethylsilyl, and t-

10   butyldiphenylsilyl, tritylmethyl, alkyl groups, acyl groups such as acetyl and propionyl, methylsulfonyl, and p-toluylsulfonyl.

The term "hydroxyalkyl," as used herein, refers to a C_1 to C_6 alkyl group in which at least one of the hydrogens attached to any of the carbon atoms is replaced with a hydroxy group.

15   The term "pharmaceutically acceptable derivative" refers to a derivative of the active compound that upon administration to the recipient, is capable of providing directly or indirectly, the parent compound, or that exhibits activity itself.

The term "pharmaceutically acceptable cation" refers to an organic or inorganic moiety that carries a positive charge and that can be administered in association with a

20   pharmaceutical agent, for example, as a countercation in a salt. Pharmaceutically acceptable cations are known to those of skill in the art, and include but are not limited to sodium, potassium, and quaternary amine.

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The term "physiologically cleavable leaving group" refers to a moiety that can be cleaved *in vivo* from the molecule to which it is attached, and includes but is not limited to an organic or inorganic anion, a pharmaceutically acceptable cation, acyl (including but not limited to (alkyl)C(O), including acetyl, propionyl, and butyryl),

5   alkyl, phosphate, sulfate and sulfonate.

The term "enantiomerically enriched composition or compound" refers to a composition or compound that includes at least 95%, and preferably at least 97, 98, 99, or 100% by weight of a single enantiomer of the compound.

The term "amino acid" includes synthetic and naturally occurring amino acids,

10   including but not limited to, for example, alanyl, valinyl, leucinyl, isoleucinyl, prolinyl, phenylalaninyl, tryptophanyl, methioninyl, glycinyl, serinyl, threoninyl, cysteinyl, tyrosinyl, asparaginyl, glutaminyl, aspartoyl, glutaoyl, lysinyl, argininyl, and histidinyl.

A "linking moiety" as used herein, is any divalent group that links two chemical

15   residues, including but not limited to alkyl, alkenyl, alkynyl, aryl, polyalkyleneoxy (for example, $-[(CH_2)_nO-]_n-$), $-C_{1-6}alkoxy-C_{1-10}alkyl-$, $-C_{1-6}alkylthio-C_{1-10}$ alkyl-, $-NR^3-$, and $-(CHOH)_nCH_2OH$, wherein n is independently 0, 1, 2, 3, 4, 5, or 6.

As explained in Chapter 2 of Thorn et al, the preparation of dithiocarbamates is very simple. The compounds of the formula R_1R_2NCSSH or R_1R_2NSSNa can be

20   formed by reaction of carbon disulfide with a secondary amine, typically in alcoholic or aqueous solution. The usual practice is to carry out this reaction in the presence of NaOH, so that the sodium dithiocarbamate salt is formed. Thus, for example, sodium

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dimethyl dithiocarbamate is formed from CS₂NaOH and dimethylamine. See Thorn et

al, page 14, and the references cited therein. Other typical dithiocarbamic compounds

disclosed and characterized in Thorn et al include: N-methyl, N-

ethyldithiocarbamates, hexamethylenedithiocarbamic acid, sodium di(beta-

5   hydroxyethyl) dithiocarbamate, sodium N-methyl, N-cyclobutylmethyl

dithiocarbamate, sodium N-allyl-N-cyclopropylmethyl-dithiocarbamate,

cyclohexylamyldithiocarbamates, dibenzyl-dithiocarbamates, sodium dimethylene-

dithiocarbamate, various pentamethylene dithiocarbamate salts, sodium pyrrolidine-N-

carbodithioate, sodium piperidine-N-carbodithioate, sodium morpholine-N-carbo-

10   dithioate, alpha-furfuryl dithiocarbamates and imidazoline dithiocarbamates.

B)   Probucol and its Derivatives

Probucol is chemically related to the widely used food additives 2,[3]-tert-

butyl-4-hydroxyanisole (BHA) and 2,6-di-tert-butyl-4-methyl phenol (BHT). Its full

15   chemical name is 4,4'-(isopropylidenedithio) bis(2,6-di-tert-butylphenol). U.S. Patent

No. 5,262,439 to Parthasarathy, incorporated herein by reference, discloses soluble

analogs of probucol in which one or both of the hydroxyl groups are replaced with

ester groups that impart water solubility to the compound. In one embodiment, the

soluble derivative is selected from the group consisting of a mono- or di- succinic acid

20   ester, glutaric acid ester, adipic acid ester, suberic acid ester, sebacic acid ester, azelaic

acid, or maleic acid ester of probucol. In another embodiment, the probucol derivative

is a mono- or di- ester in which the ester contains an alkyl or alkenyl group that

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contains a functionality selected from the group consisting of a carboxylic acid group.

Any of the compounds described in the '439 patent can be used in this invention.

U.S. Patent No. 5,155,250, also incorporated herein by reference, discloses that 2,6-dialkyl-4-silylphenols are antiatherosclerotic agents. The same compounds are

5 disclosed as serum cholesterol lowering agents in PCT Publication No. WO 95/15760, published on June 15, 1995. U.S. Patent No. 5,608,095, incorporated by reference, discloses that alkylated-4-silyl-phenols inhibit the peroxidation of LDL, lower plasma cholesterol, and inhibit the expression of VCAM-1, and thus are useful in the treatment of atherosclerosis. Any of these compounds can also be used in this

10 invention.

C)   N-Acetyl Cysteine and its Derivatives

Cysteine is an amino acid with one chiral carbon atom. It exists as an L-enantiomer, a D-enantiomer, or a racemic mixture of the L- and D-enantiomers. The

15 L-enantiomer is the naturally occurring configuration.

N-acetylcysteine (acetamido-mercaptopropionic acid, NAC) is the N-acetylated derivative of cysteine. It also exists as an L-enantiomer, a D-enantiomer, an enantiomerically enriched composition of one of the enantiomers, or a racemic mixture of the L and D enantiomers. The term "enantiomerically enriched

20 composition or compound" refers to a composition or compound that includes at least 95%, and preferably, at least 97% by weight of a single enantiomer of the compound. Any of these forms of NAC can be delivered as an antioxidant in the present

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invention. In one embodiment, a single isomer of a thioester or thioether of NAC or its salt, and most preferably, the naturally occurring L-enantiomer, is used in the treatment process.

N-acetylcysteine exhibits antioxidant activity (Smilkstein, Knapp, Kulig and Rumack, *N. Engl. J. Med.* 1988, Vol. 319, pp. 1557-62; Knight, K.R., MacPhadyen, K., Lepore, D.A., Kuwata, N., Eadie, P.A., O'Brien, B. *Clinical Sci.*, 1991, Vol. 81, pp. 31-36; Ellis, E.F., Dodson, L.Y., Police, R.J., *J. Neurosurg.*, 1991, Vol. 75, pp. 774-779). The sulfhydryl functional group is a well characterized, highly reactive free radical scavenger. N-acetylcysteine is known to promote the formation of glutathione (a tri-peptide, also known as g-glutamylcysteinylglycine), which is important in maintaining cellular constituents in the reduced state (Berggren, M., Dawson, J., Moldeus, P. *FEBS Lett.*, 1984, Vol. 176, pp. 189-192). The formation of glutathione may enhance the activity of glutathione peroxidase, an enzyme which inactivates hydrogen peroxide, a known precursor to hydroxyl radicals (Lalitha, T., Kerem, D., Yanni, S., *Pharmacology and Toxicology*, 1990, Vol.66, pp. 56-61)

N-acetylcysteine exhibits low toxicity *in vivo*, and is significantly less toxic than deprenyl (for example, the LD_{50} in rats has been measured at 1140 and 81 mg/kg intravenously, for N-acetylcysteine and deprenyl, respectively).

N-acetyl cysteine and derivatives thereof are described, for example, in WO/95/26719. Any of the derivatives described in this publication can be used in accordance with this invention.

D)   Scavengers of Peroxides, including but not limited to catalase and

pyruvate

E)   Thiols including dithiothreitol and 2-mercaptoethanol

F)   Antioxidants which are inhibitors of lipid peroxidation, including but

not limited to Trolox™, BHA, BHT, aminosteroid antioxidants, tocopherol and its

analogs, and lazaroids

G)   Dietary antioxidants, including antioxidant vitamins (vitamin C or E or

synthetic or natural prodrugs or analogs thereof), either alone or in combination with

each other, flavanoids, phenolic compounds, caratenoids, and alpha lipoic acid

H)   Inhibitors of lipoxygenases and cyclooxygenases, including but not

limited to nonsteriodal antiinflammatory drugs, COX-2 inhibitors, aspirin-based

compounds, and quercetin

I)   Antioxidants manufactured by the body, including but not limited to

ubiquinols and thiol antioxidants, such as, and including glutathione, Se, and lipoic

acid

J)   Synthetic Phenolic Antioxidants: inducers of Phase I and II drug-

metabolizing enzymes

III. ANTINEOPLASTIC AGENTS

The term "antineoplastic agents," as used herein, refers to any substance that

decreases abnormal cell proliferation. Antineoplastic agents have been described

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extensively in a number of texts, including Martindale, *The Extra Pharmacopoeia*, 31ˢᵗ

Edition, Royal Pharmaceutical Society (1996).

Antineoplastic agents include:

(i) antifolates;

5   (ii) antimetabolites (including purine antimetabolites, cytarabine, fudarabine,

floxuridine, 6-mercaptopurine, methotrexate, 5-fluoropyrimidine, including 5-

fluorouracil, cytidine analogues such as β-L-1,3-dioxolanyl cytidine and 6-

thioguanine);

(iii) hydroxyurea;

10   (iv) mitotic inhibitors (including CPT-11, Etoposide(VP-21)), taxol, and

vincristine,

(v) alkylating agents (including but not limited to busulfan, chlorambucil,

cyclophosphamide, ifofamide, mechlorethamine, melphalan, and thiotepa);

(vi) nonclassical alkylating agents, platinum containing compounds,

15   bleomycin, anti-tumor antibiotics, anthracycline, anthracenedione, topoisomerase 11

inhibitors, hormonal agents (including but not limited to corticosteroids

(dexamethasone, prednisone, and methylprednisone); and

(v) androgens such as fluoxymesterone and methyltestosterone, estrogens such

as diethylstilbesterol, antiestrogens such as tamoxifen, LHRH analogues such as

20   leuprolide, antiandrogens such as flutamide, aminoglutethimide, megestrol acetate,

and medroxyprogesterone), asparaginase, carmustine, lomustine, hexamethyl-

melamine, dacarbazine, mitotane, streptozocin, cisplatin, carboplatin, levamasole, and

leucovorin.

A more comprehensive list of antineoplastic agents includes Aceglatone;

Aclarubicin; Altretamine; Aminoglutethimide; 5-Aminogleavulinic Acid; Amsacrine;

5   Anastrozole; Ancitabine Hydrochloride; 17-1A Antibody; Antilymphocyte

Immunoglobulins; Antineoplaston A10; Asparaginase; Pegaspargase; Azacitidine;

Azathioprine; Batimastat; Benzoporphyrin Derivative; Bicalutamide; Bisantrene

Hydrochloride; Bleomycin Sulphate; Brequinar Sodium; Broxuridine; Busulphan;

Campath-IH; Caracemide; Carbetimer; Carboplatin; Carboquone; Carmofur;

10   Carmustine; Chlorambucil; Chlorozotocin; Chromomycin; Cisplatin; Cladribine;

Corynebacterium parvum; Cyclophosphamide; Cyclosporin; Cytarabine; Dacarbazine;

Dactinomycin; Daunorubicin Hydrochloride; Decitabine; Diaziquone;

Dichlorodiethylsulphide; Didemnin B.; Docetaxel; Doxifluridine; Doxorubicin

Hychloride; Droloxifene; Echinomycin; Edatrexate; Elliptinium; Elmustine;

15   Enloplatin; Enocitabine; Epirubicin Hydrochloride; Estramustine Sodium Phosphate;

Etanidazole; Ethoglucid; Etoposide; Fadrozole Hydrochloride; Fazarabine;

Fenretinide; Floxuridine; Fludarabine Phosphate; Fluorouracil; Flutamide;

Formestane; Fotemustine; Gallium Nitrate; Gencitabine; Gusperimus;

Homoharringtonine; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine;

20   Improsulfan Tosylate; Inolimomab; Interleukin-2; Irinotecan; JM-216; Letrozole;

Lithium Gamolenate; Lobaplatin; Lomustine; Lonidamine; Mafosfamide; Melphalan;

Menogaril; Mercaptopurine; Methotrexate; Methotrexate Sodium; Miboplatin;

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Miltefosine; Misonidazole; Mitobronitol; Mitoguazone Dihydrochloride; Mitolactol;

Mitomycin; Mitotane; Mitozanetrone Hydrochloride; Mizoribine; Mopidamol;

Multialchilpeptide; Muromonab-CD3; Mustine Hydrochloride; Mycophenolic Acid;

Mycophenolate Mofetil; Nedaplatin; Nilutamide; Nimustine Hydrochloride;

5   Oxaliplatin; Paclitaxel; PCNU; Penostatin; Peplomycin Sulphate; Pipobroman;

Pirarubicin; Piritrexim Isethionate; Piroxantrone Hydrochloride; Plicamycin; porfimer

Sodium; Prednimustine; Procarbazine Hydrochloride; Raltitrexed; Ranimustine;

Razoxane; Rogletimide; Roquinimex; Sebriplatin; Semustine; Sirolimus; Sizofiran;

Sobuzoxane; Sodium Bromebrate; Sparfosic Acid; Sparfosate Sodium; Sreptozocin;

10   Sulofenur; Tacrolimus; Tamoxifen; Tegafur; Teloxantrone Hydrochloride;

Temozolomide; Teniposide; Testolactone; Tetrasodium Meso-

tetraphenylporphinesulphonate; Thioguanine; Thioinosine; Thiotepa; Topotecan;

Toremifene; Treosulfan; Trimetrexate; Trofosfamide; Tumor Necrosis Factor;

Ubenimex; Uramustine; Vinblastine Sulphate; Vincristine Sulphate; Vindesine

15   Sulphate; Vinorelbine Tartrate; Vorozole; Zinostatin; Zolimomab Aritox; and

Zorubicin Hydrochloride.

IV. ABNORMAL CELL HYPERPROLIFERATIVE CONDITIONS

Antioxidants can be used to increase the cytotoxicity of antineoplastic agents to

20   disorders of abnormal cellular proliferation, including, but not limited to:

benign tumors, including, but not limited to papilloma, adenoma, firoma,

chondroma, osteoma, lipoma, hemangioma, lymphangioma, leiomyoma,

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rhabdomyoma, meningioma, neuroma, ganglioneuroma, nevus, pheochromocytoma, neurilemona, fibroadenoma, teratoma, hydatidiform mole, granuosa-theca, Brenner tumor, arrhenoblastoma, hilar cell tumor, sex cord mesenchyme, interstitial cell tumor, and thyoma;

5   malignant tumors (cancer), including but not limited to carcinoma, including renal cell carcinoma, prostatic adenocarcinoma, bladder carcinoma,and adenocarcinoma, fibrosarcoma, chondrosarcoma, osteosarcoma, liposarcoma, hemangiosarcoma, lymphangiosarcoma, leiomyosarcoma, rhabdomyosarcoma, myelocytic leukemia, erythroleukemia, multiple myeloma, glioma, meningeal

10   sarcoma, thyoma, cystosarcoma phyllodes, nephroblastoma, teratoma choriocarcinoma, cutaneous T-cell lymphoma (CTCL), cutaneous tumors primary to the skin (for example, basal cell carcinoma, squamous cell carcinoma, melanoma, and Bowen's disease), breast and other tumors infiltrating the skin, Kaposi's sarcoma, and premalignant and malignant diseases of mucosal tissues, including oral, bladder, and

15   rectal diseases, central nervous system tumors (glioblastomas), meningiomas, and astrocytomas;

     hyperproliferative and preneoplastic lesions, including mycosis fungoides, psoriasis, dermatomyositis, rheumatoid arthritis, viruses (for example, warts, herpes simplex, and condyloma acuminata), molluscum contagiosum, remalignant and

20   malignant diseases of the female genital tract (cervix, vagina, and vulva).

     Of these, particular conditions which can be treated using this method include colorectal cancer, ovarian cancer, bone cancer, renal cancer, breast cancer, gastric

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cancer, pancreatic cancer, melanoma, hematopoietic tumors such as lymphoma, leukemia, plasma cell dyscrasias, and multiple meyloma and amylodosis.

Antioxidants can also be used in combination with antineoplastic agents to treat cardiovascular proliferative disease such as post-angioplasty restenosis and

5   atherosclerosis.

V. PHARMACEUTICAL COMPOSITIONS

A host, including mammals, and specifically humans, suffering from any of the above-described conditions can be treated by the topical or systemic administration

10   to the patient of an effective amount of an antioxidant, optionally in combination with an antineoplastic agent, in the presence of a pharmaceutically acceptable carrier or diluent. The antioxidant can be administered prior to, in combination with, or following treatment with an antineoplastic agent when used to increase the cytotoxic effect of the antineoplstic agent. Methods and dosages for the administration of

15   antineoplastic agents are known to those skilled in the art, and are described in a number of texts, including the *The Physician's Desk Reference*, Martindale's *The Extra Pharmacopeia*, and Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, or can be easily determined using standard methods.

The antioxidant can be administered subcutaneously, intravenously,

20   intraperitoneally, intramuscularly, parenterally, orally, submucosally, by inhalation, transdermally via a slow release patch, or topically, in an effective dosage range to treat the target condition. Typical systemic dosages for all of the herein described

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conditions are those ranging from 0.01 mg/kg to 500 mg/kg of body weight per day as

a single daily dose or divided daily doses. Typical dosages for topical application are

those ranging from 0.001 to 100% by weight of the active compound.

The compound is administered for a sufficient time period to alleviate the

5   undesired symptoms and the clinical signs associated with the condition being treated.

The active compound is included in the pharmaceutically acceptable carrier or

diluent in an amount sufficient to deliver to a patient a therapeutic amount of

compound *in vivo* in the absence of serious toxic effects.

The concentration of active compound in the drug composition will depend on

10   absorption, inactivation, and excretion rates of the drug as well as other factors known

to those of skill in the art. It is to be noted that dosage values will also vary with the

severity of the condition to be alleviated. It is to be further understood that for any

particular subject, specific dosage regimens should be adjusted over time according to

the individual need and the professional judgment of the person administering or

15   supervising the administration of the compositions, and that the dosage ranges set

forth herein are exemplary only and are not intended to limit the scope or practice of

the claimed composition. The active ingredient may be administered at once, or may

be divided into a number of smaller doses to be administered at varying intervals of

time.

20   A preferred mode of administration of the active compound for systemic

delivery is oral. Oral compositions will generally include an inert diluent or an edible

carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the

purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition.

5   The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon

10   dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit,

15   for example, coatings of sugar, shellac, or other enteric agents.

The compound or its salts can be administered as a component of an elixir, suspension, syrup, wafer, lozenge, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

20   The compound can also be mixed with other active materials which do not impair the desired action, or with materials that supplement the desired action, such as

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antibiotics, antifungals, antiinflammatories, antivirals, or other immunosuppressive agents.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

If administered intravenously, preferred carriers are physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS).

In a preferred embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and

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Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to

infected cells with monoclonal antibodies to viral antigens) are also preferred as

pharmaceutically acceptable carriers. These may be prepared according to methods

known to those skilled in the art, for example, as described in U.S. Patent No.

5   4,522,811 (which is incorporated herein by reference in its entirety). For example,

liposome formulations may be prepared by dissolving appropriate lipid(s) (such as

stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl

phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated,

leaving behind a thin film of dried lipid on the surface of the container. An aqueous

10   solution of the compound is then introduced into the container. The container is then

swirled by hand to free lipid material from the sides of the container and to disperse

lipid aggregates, thereby forming the liposomal suspension.

Suitable vehicles or carriers for topical application can be prepared by

conventional techniques, such as lotions, suspensions, ointments, creams, gels,

15   tinctures, sprays, powders, pastes, slow-release transdermal patches, suppositories for

application to rectal, vaginal, nasal or oral mucosa. In addition to the other materials

listed above for systemic administration, thickening agents, emollients, and stabilizers

can be used to prepare topical compositions. Examples of thickening agents include

petrolatum, beeswax, xanthan gum, or polyethylene, humectants such as sorbitol,

20   emollients such as mineral oil, lanolin and its derivatives, or squalene. A number of

solutions and ointments are commercially available, especially for ophthalmic

applications.

VI. ILLUSTRATIVE EXAMPLES

The following examples are provided for the purpose of illustrating various embodiments of the invention and are not intended to limit the scope of the present invention.

EXAMPLE 1

HCT 116 and HCT 15 human CRC cells were obtained from the American type Culture Collection. p21$^{WAF1/CIP1}$-/- cancer cells generated from HCT 116 cells by T. Waldman were provided by J. Pietenpol (Vanderbilt University, TN) and HPV E6-transfected HCT 116 by W. S. El-Deiry (University of Pennsylvania, PA) [W.S. El-Deiry et al., *Cell* 75, 817 (1993)]. All cancer cell lines used in these studies were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL) with high glucose and supplemented with 10% heat-activated fetal bovine serum (FBS), non-essential amino acids, L-glutamine, and penicillin G Sodium (100 U/ml) and streptomycin sulfate (100 mg/ml) at 37°C in 5%CO_2 in air.

To determine the effect of pyrrolidinedithiocarbamate (Sigma Chemical Co., MA), a vitamin E analogue (6-hydroxy-2.5,7,8-tetramethylchroman-2-carboxylic acid: vit (E) (Aldrich), 5-FU (Hoffmann-LaRoche Inc. Nutley, NJ) or doxorubicin (Sigma) on anchorage-independent growth, HCT 15 and HCT 116 cells were plated at 10 X10^3 cells/35 mm plate in DMEM supplemented with 1% FBS and 0.4% agar along with the factor to be tested. The number of colonies was quantified under 10 days using the Omnicon image analyzer. Colonies greater than 50 microns in diameter

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(approximately 50 cells) were scored as positive after 10 days. Preliminary studies indicated that pyrrolidinedithiocarbamate and vitamin E had no significant effect on CRC cell plating efficiency, at concentrations between 25-200 μM and 0.1-10 mM, respectively. Higher concentrations resulted in non-specific cytotoxicity.

EXAMPLE 2

DNA Content of nuclei was determined as described [I.G. Nicoletti *et al., J. Immunol. Methods.* **139**, 271 (1991)] by lysing plasma membranes, staining nuclear DNA with propidium iodide (50 mg/ml), and quantitating the relative DNA content of nuclei using the Becton Dickinson FACSORT fluorescence-activated cell sorter. The proportion of nuclei in each phase of the cell cycle was determined using MODFIT - DNA analysis software. Detection of apoptotic cells either by fluorescence microscopy or by flow cytometry was performed using the ApopTag Plus In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD) as described in the manufacturer's protocol. Briefly, gigoxygenin-labeled nucleotides were added to free 3'OH groups of DNA produced by DNA fragmentation during apoptosis by terminal deoxynucleotidyl transferase (TdT). Digoxygenin was detected by a FTC-conjugated anti-digoxygenin antibody. Analysis was carried out using the fluorescence-activated cell sorter and FITC staining visualized using a fluorescence microscope (Zeiss).

EXAMPLE 3

Intracellular H_2O_2 levels were analyzed by flow cytometry using dihydrorhodamine 1234 (DHR) as a specific fluorescent dye probe [G. Rothe, A. Emmendorffer, A. Oser, J. Roesler, G. Valet, *J. Immun. Methods* **138**, 133 (1991); J.

A. Royall, H. Ischiropoulos, *Arch. Biochem. Biophysics* 302, 348 (1993)]. CRC cells were grown in DMEM containing 1 mM DHR and pyrrolidinedithiocarbamate (70 μM) or vit E (3 mM) for up to 24 hours. Following trypinization, trypsin activity was quenched with 2% FBS in phosphate buffered saline and cells fixed in 1% paraformaldahyde (Sigma). Cellular rhodamine 123 fluorescence intensity of 1×10^4 cells was measured for each sample using a Becton-Dickenson FACS Vantage flow cytometer with the excitation source at 488 nm and emission wave length of 580 nm. Histograms were analyzed with the software program PC-Lysis (Becton Dickenson). Background fluorescence from blank wells was subtracted from each reading.

EXAMPLE 4

Male athymic Balb/c *nu/nu* mice were obtained from the Harlan Sprague-Dawley Company at 4-6 weeks of age and were quarantined for at least 2 weeks before the study. Animal experiments were carried out in accordance with both institutional and federal animal care regulations. HCT 116 and HCT 15 CRC cell lines were grown in DMEM supplemented with 10% FBS as described above. Cells were harvested through two consecutive trypsinizations, centrifuged at 300g for 5 minutes, washed twice, and resuspended in sterile phosphate buffered saline. One X 10^6 cells in 0.2 ml were injected subcutaneously between the scapula of 7- to 10-week-old male nude mice.

EXAMPLE 5

Tumor volumes were estimated weekly by measuring the maximal length, width, and height. Once tumors reached a mean size of 120 to 150 mm^3, animals

received either weekly i.p. injections of pyrrolidinedithiocarbamate (70 µM) or

vitamin E (3 mM), 5-FU (40 mg/kg) or saline, or a combination of

pyrrolidinedithiocarbamate or vitamin E and 5-FU or 6 weeks. In cross-over

experiments, animals received the above treatments for three weeks (with the

5   exception of vitamin E), and then were crossed-over to either the combination

treatment of pyrrolidinedithiocarbamate and 5-FU (saline, pyrrolidinedithiocarbamate

or 5-FU alone) or discontinued treatment pyrrolidinedithiocarbamate and 5-FU) for

the remaining three weeks of the experiment. In preliminary experiments, a series of

single doses of pyrrolidinedithiocarbamate, vitamin E or 5-FU were administered over

10   a 30 day period to establish LD_{50} and effective route of administration (data not

shown). Tumor volumes were recorded weekly until termination of the study.

EXAMPLE 6

Tumor tissues were fixed overnight in 4% (v/v) paraformaldehyde and

embedded in paraffin according to standard histological procedures. BrDU staining

15   was performed as described [Holmgren, et al., Nature Med. 1, 149 (1955)]. TdT

labeling of fragmented DNA (TUNEL) was performed as described. The proliferative

index (BrDU) and the apoptotic index (TUNEL) were estimated by the percentage of

cells scored under a microscope at 200-fold magnification. The proliferative indices

for HCT 116 and HCT 15-derived tumors (irrespective of treatments) were 53.1 ± 5.2

20   and 63.1± 7.2, respectively.

EXAMPLE 7

For Western blot analysis, cells were lysed in 50 mM Tris-Cl. pH7.4, 300 mM NaCl, 2 mM EDTA, 0.5% Nonidet-40, 05 mM phenylmethysulfonyl fluoride aprotinin (1 µg/ml), pepstatin (1 µg/ml), and leupeptin (2 µg/ml). One hundred mg of extract (as determined by Bradford analysis) was applied to 12% SDS-PAGE gels and transferred to 0.2 µM pore nitrocellulose membranes (Schleicher and Schuell). Blots were probed with antibodies raised against p21 WAF1/CIPI, p53, p27 or C/EBPβ (Santa Cruz) at a final concentration of 0.1 µg/ml. After washing, blots were incubated with donkey-anti-rabbit or goat-anti-mouse IgG-horseradish peroxidase conjugates, and developed using Enhanced Chemiluminescence (amersham, Arlington Heights, IL).

EXAMPLE 8

RNA was extracted as described [M. Schwab, K. Alitalo, H. E. Varmus, J.M. Bishop, *Nature (Lond.)* **303**, 497 (1983)]. Poly (A)$^+$ mRNA was separated by electrophoresis through 1% (w/v) agaroseformaldehyde gels, and northern blotting was performed as previously described (Coffey, et al., *Cancer Res.* **47**, 4590 (1987)]. A human p21$^{WAF1/CIPI}$ cDNA probe was provided by B. vogelstein (John Hopkins Oncology Center, Baltimore, MD) and labeled with [^{32}P]dCTP by the random primer extension method. Hybridization and posthybridization washes were carried out at 43°C. IB15 was used as a control for equivalent loading and transfer [P. E. Danielson, *et al.*, DNA **7**, 261 (1988)].

EXAMPLE 9

The human p21$^{WAF1/CIP1}$ promoter construct (WWP-luc) was provided by B. Vogelstein [W. El-Deiry, *et al., Cell* 75, 817 (1993)]. CRC cell lines were grown to 50% confluence prior to transfection with CELLFECTIN per manufacturer's instructions (GIBCO BRL). For all luciferase assays, total DNA transfected was kept constant with addition of pBSKII$^+$ or pCMV-basic. All pCMV-C/EBP expression vectors were provided by L. Sealy (Vanderbilt University, TN). pCMV-CAT was transfected as an internal control for gene expression. At 12 hours posttransfection, selected cells were treated with 70 uM pyrrolidinedithiocarbamate. After 24 hours of treatment, cell lysates were prepared and luciferase activity was assayed as described [A. Misra-Press, C. S. Rim, H. Yao, M. S. Roberson, P. J. S. Stork, *J. Biol. Chem.* 270, 14587 (1995)]. Luciferase activity was normalized to CAT activity, and results were reported as fold activation above basal levels.

EXAMPLE 10

The 2.4 kilobase pair genomic gragment containg the p21$^{WAF1/CIP1}$ cDNA start site at its 3' end was subcloned into the *Hind* III site of the luciferase reporter vector, pGL2-basic (Promega). p21$^{WAF1/CIP1}$ deletion mutants (D2198 to D1138) were generated by PCR using internal p21$^{WAF1/CIP1}$ primers designed against the published p21$^{WAF1/CIP1}$ promoter sequence (GenBank). In each case, PCR products were subcloned into pGL2-basic and the sequences verified by double-stranded DNA sequencing. Mutagenesis of the NF_IL6 recognition site was performed using the Muta-Gene M13 *In vitro* mutagenesis kit (Bio-Rad, Hercules, CA).

The presence of the desired TT to AA base pair change was verified by DNA sequencing.

EXAMPLE 11

Complementary oligonucleotides corresponding to bases -1884 through -1904
in the wild type and the NF_IL6 mutant p21$^{WAF1/CIP1}$ promoter suquence were
synthesised (wild type, GTACTTAAGAAATATTGAAT and
ATTCAATATTTCTTAAGTAC; mutant; GTACAAAAGAAATATTGAAT and
ATCAATATTTCTTTTGTAC). Two hundred ng of each oligo was end-labeled with
200 µCi γ-^{32}P-labeled ATP and T4 polynucleotide kinase. The resulting end-labeled
oligos were then annealed and gel purified. Preparation of nuclear extract from CRC
cells treated with antioxidants and the conditions for electrophoretic shift mobility
assays (EMSA) were as described [Kailoff, et al., *Science* 253, 786 (1991)]. When
antisera were added, nuclear extracts and 2 µl of C/EBP α, β, or δ polyclonal antibody
(Santa Cruz) were incubated for 10 minutes at room temperature before the addition of
the radiolabeled probe.

EXAMPLE 12

Two human colorectoal cancer cell lines, HCT 116 (wild type p53) and HCT
15 (mutant p53), were treated with increasing amounts of either
pyrrolidinedithiocarbamate or vitamin E in a soft agar *ex vivo* model of
tumorigenicity. Both pyrrolidinedithiocarbamate and vitamin E caused a dose-
dependent reduction in anchorage-independent growth of HCT 116 and HCT 15 cells
(Figure 1A). This analysis was extended to a variety of tumor cell lines derived from

the colon (HCA-7, Difi, RKO, SW620), breast (MCF-7, MDA-MB231), and stomach (Hs 746T). At these concentratons, both antioxidants were effective in inhibiting anchorage-independent growth of all tumor cell lines tested, independent of their p53 status (Difi, RKO, data not shown).

5   Treatment of HCT 116 or HCT 15 CRC cells with either pyrrolidinedithiocarbamate (70 µM) or vitamin E (3 mM) for 24 hours, followed by propidium iodide staining of cells and subsequent flow cytometric analysis, revealed that both compounds induced a significant accumulation of cells in the G_1 peak, suggesting that the observed growth inhibitory effects of pyrrolidinedithiocarbamate

10   or vitamin E in soft agar were due to cell cycle arrest and/or apoptosis (Figure 1B). To determine whether these cell cycle perturbations could be correlated with the antioxidant properties of these compounds, both the intracellular redox status (by endogenous H_2O_2 levels) and the percentage of cells undergoing G_1 cell cycle arrest or apoptosis (by flow 25cytrometric analysis) were quantified over a 24 hour period in

15   antioxidant-treated cells.

As shown in Figure 1C, both pyrrolidinedithiocarbamate and vitamin E significantly reduced endogenous H_2O_2 levels in both cell lines, with pyrrolidinedithiocarbamate being the more effective reducing agent. Furthermore, this decrease in H_2O_2 levels correlated with the induction of G_1 cell cycle arrest and the

20   appearance of TUNEL-positive nuclei in these cells. Treatment of HCT 15 cells with the membrane-permeant antioxidant N-acetyl-L-cysteine (NAC) and the dietary antioxidant, vitamin C, showed a similar reduction in H_2O_2 levels and the induction of

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apoptosis (Figure 1D), supporting a role for reactive oxygen species in cell cycle

progression. Since antioxidants may alter the intracellular redox milieu

through reactive oxygen species other than H_2O_2, HCT 15 cells were transiently

transfected with an expression plasmid encoding human catalase. Overexpression of

5   catalase markedly reduced H_2O_2 levels and induced cell cycle arrest and apoptosis in

these cells, thus directly implicating H_2O_2 as an important mediator of the observed

cell cycle effects in these antioxidant-treated cells.

To demonstrate further that antioxidants enhance the cytotoxic efficacy of 5-FU

and doxorubicin, the IC_{50} value of each drug was determined for HCT 116 and HCT

10   15 cells grown in soft agar in the presence or absence of 70 μM

pyrrolidinedithiocarbamate or 3 mM vitamin E (the approximate IC_{50} values for these

compounds in both cell lines). Pyrrolidinedithiocarbamate or vitamin E decreased the

IC_{50} for both 5-FU and doxorubicin compared to cells treated with either of the

individual drugs alone (Figure 1E). These effects were more pronounced with

15   pyrrolidinedithiocarbamate, perhaps reflecting its more potent reducing ability.

Mechanisms of cellular uptake and metabolism of 5-FU and doxorubicin differ

significantly. Thus, it is unlikely that pyrrolidinedithiocarbamate or vitamin E

modulate the cytotoxicity of 5-FU or doxorubicin via alterations in these pathways.

EXAMPLE 13

20   The therapeutic efficacy of pyrrolidinedithiocarbamate or vitamin E was next

examined *in vivo* by growing HCT 116 or HCT 15 cells as tumor zenografts in

athymic mice. Agter establishment of palable tumors (mean tumor volume 150 mm^3),

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animals either received weekly i.p. injections of pyrrolidinedithiocarbamate, vitamin E, and/or 5-FU or saline as a negative control. The results with HCT 116 cells are shown in Figure 2A. After 4 weeks, tumor volumes in control mice necessitated sacrifice in accordance with institutional protocol. Individually,

5   pyrrolidinedithiocarbamate, vitamin E and 5-FU significantly reduced tumor volume over the 6-weeks compared to saline-treated controls. Addition of pyrrolidinedithiocarbamate or vitamin E significantly enhanced the effect of 5-FU. In all nine animals with complete abolishment of tumors, no sign of tumor 25regrowth has been observed following discontunuation of combined treatment for 2 months.

10   Similar results were seen in HCT 15-derived xenografts with the exception that the combination regimens were more effective in these mutant p53 CRC cells.

To further explore the *in vivo* efficacy of pyrrolidinedithiocarbamate and 5-FU in established HCT 15-derived tumors, mice were crossed over to combined treatment once significant differences in single agent therapies were established (Figure 2B).

15   Mice which initially received no treatment developed large tumors (2780 ± 257 mm^3) by 3 weeks. Treatment of these mice with 5-FU and pyrrolidinedithiocarbamate at this time reduced the size of even these advanced lesions (week 6: 1184 ± 96 mm^3). Cross-over to combined treatment (5-FU and pyrrolidinedithiocarbamate) also reduced tumor size in mice initially treated with a single agent. Tumors decreased in size from

20   1864 ± 190 mm^3 to 660 ± 82 mm^3 and 1325 ± 210 mm^3 to 637 ± 231 mm^3, for animals treated initially with 5-FU and pyrrolidinedithiocarbamate alone, respectively. These

results complement the *in vitro* findings, and indicate that antioxidants can

significantly enhance the efficacy of 5-FU in CRC cells.

No signs of agent-induced toxicity were observed in the mice, as judged by

changes in body weight or gross anatomical and microscopic examination of major

5   organs. At necropsy, all tumors exhibit central necrosis grossly, irrespective of tumor

size or treatment regimen. Since tumors from mice treated with pyrrolidinedithio-

carbamate and 5-FU were no longer present, this treatment group was omitted from

these analyses. Immunohistochemical analysis of residual tumors demonstrated a high

proliferative index, irrespective of the treatment regimen. However, the apoptotic

10   index increased approximately 5-fold following vitamin E, in both xenograft models

(Figure 2C). In contrast, the apoptotic index of 5-FU-treated tumors was markedly

higher in cells expressing wild type p53 (HCT 116) compared to mutant p53 (HCT

15), supporting a role for p53-mediated apoptosis in 5-FU cytotoxicity. Combined

vitamin E and 5-FU treatment was able to further increase the apoptotic index in these

15   tumors, even in a mutant p53 genetic background. The apparent synergy between

vitamin E and 5-FU-induced apoptosis in mutant p53 HCT 15 cells suggests that

antioxidants may re-establish the apoptotic signaling pathway.

EXAMPLE 14

Regulation of G_1 cell cycle arrest and subsequent apoptosis has been attributed

20   to a number of cellular proteins, including p53 and the cyclin-dependent kinase

inhibitors, such as p21[WAF1/CIP1] and p27. Pyrrolidinedithiocarbamate had no effect on

p53 or p27 protein levels in either HCT 116 or HCT 15 cells over a 24 hour period, as

determined by Western blot analysis (Figure 3A). In contrast, p21$^{WAFI/CIPI}$ protein and

mRNA levels increased within one hour after pyrrolidinedithiocarbamate treatment

and persisted for 24 hours (Figure 1B).

Induction of p21$^{WAFI/CIPI}$ mRNA by pyrrolidinedithiocarbamate appeared to be

5   p53 independent, as the antioxidant effect was not attenuated in HCT 116 cells

expressing human papillomavirus (HPV) E6, which inactivates p53 through ubiquitin-

mediated protein degradation (Figure 3B) (Scheffner, et al, *Proc. Natl.Acad.* Sci.

U.S.A. **88**, 5523 (1991); Crook, et al, *Oncogene* **6**, 873 (1991)). Similar increases in

p21$^{WAFI/CIPI}$ expression were observed in HCT 116 and HCT 15 cells treated with

10   vitamin E.

To confirm that the induction of p21$^{WAFI/CIPI}$ by antioxidants was required for

these cell cycle disruptions, parental HCT 116 cells or cells were treated with a

targeted disruption of p21$^{WAFI/CIPI}$ by pyrrolidinedithiocarbamate or vitamin E for 24

hours (Fig. 3C). In both cells types, there was a significant attenuation of antioxidant-

15   mediated apoptosis, suggesting that p21$^{WAFI/CIPI}$ plays a pivotal role in antioxidant-

mediated cell death.

EXAMPLE 15

To confirm that induction of p21$^{WAFI/CIPI}$ was dependent on the transcriptional

activity of antioxidants, HCT 116 and HCT 15 cells were transfected with a 2.4

20   kilobase pair fragment of the p21$^{WAFI/CIPI}$ promoter linked to a luciferase reporter gene.

Treatment of transfected cells with pyrrolidinedithiocarbamate led to an approximate

five-fold induction of the p21$^{WAFI/CIPI}$ promoter activity in both HCT 116 and HCT 15

cells, again consistent with a p53-independent induction of p21$^{WAF1/CIP1}$ mRNA and

protein (Figure 4A). Serial deletions of this promoter demonstrated that the

pyrrolidinedithiocarbamate responsive element(s) of the p21$^{WAF1/CIP1}$ promoter was

located between nucleotide-2078 and -1874. Disruption of this site by site-directed

5   mutagenesis abolished pyrrolidinedithiocarbamate induction of luciferase activity,

demonstrating that the NF_IL6 site is required for pyrrolidinedithiocarbamate-induced

p21$^{WAF1/CIP1}$ transcription.

The NF_IL6 consensus sequence is recognized by members of the

CCAAT/enhancer binding protein (C/EBP) family of transcription factors (S. Akira

10   and T. Kishimoto, *Immunol Rev.* 127, 25 (1992); Landschulz *et al., Genes Dev.* 2 786

(1988); Cao, et al., *ibid.* 5, 1538 (1991); Chang, et al., *Mol. Cell. Biol.* 10, 6642

(1990); Williams *et al.,* ibid. 5, 1553 (1991); Akira *et al.,* EMBO. J.9, 1897 (1990);

Poli, et al., *Cell* 63, 25 643 (1990)). These factors contain a basic DNA-binding

region adjacent to a leucine zipper (bZIP) dimerization domain facilitating the

15   formation of homodimers or heterodimers with other bZIP proteins. Interestingly,

C/EBPα has been shown to transcriptionally upregulate p21$^{WAF1/CIP1}$ and inhibit

cellular proliferation in mouse preadipocytes, although no correlation was shown with

apoptosis.

DNA binding activity to the p21$^{WAF1/CIP1}$ NF_IL6 site was increased following

20   pyrrolidinedithiocarbamate treatment, as determined by electrophoretic mobility shift

assays (EMSA) performed with a ^{32}P-labeled oligonucleotide containing the

p21$^{WAF1/CIP1}$ NF_IL6 *cis* element and nuclear extracts from HCT 116 and HCT 15 cells

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treated over a 24 hour period (Figure 4B: left panel). Shifted complexes were

competed by 50-fold molar excess of an unlabeled oligonucleotide containing a

consensus NF_IL6 sequence (right panel: lane 2), but not by an oligonucleotide

containing a mutated NF_IL6 consensus sequence (lane 3), indicating that the induced

5   complex was specific for the NF_IL6 *cis* element. Supershift analysis of the induced

complex suggested that shifted complexes were due to the interaction of C/EPBβ (lane

5) with the NF_IL6 *cis* element and not C/EBPα (lane 4) or C/EBPδ (lane 6).

To confirm that C/EBPβ could influence p21$^{WAF1/CIP1}$ transcriptional activity, a

eukaryotic expression plasmid encoding C/EBPα,β or δ was contransfected into either

10   HCT 116 or HCT 15 cells with the full length p21$^{WAF1/CIP1}$- luciferase promoter

construct (Figure 4C and 4D). Transfection of C/EBPβ strongly activated p21$^{WAF1/CIP1}$

promoter activity in a dose-dependent manner, and mutation of the NF_IL6 site

abolished this stimulation. In contrast, C/EBPα or C/EBPδ failed to stimulate p21

promoter activity.

15   Finally, the functional role of C/EBPβ was examined in the apoptotic signaling

pathway by generating lines of HCT 15 cells that were stably transfected with the

human C/EBPβ cDNA, in both the sense and antisense orientation, under the control

of an ecdysone inducible promoter. To avoid the possibility that constitutively

expressed C/EBPβ might induce cell death, and ecdysone (muristerone A)-inducible

20   expression system (Invitrogen, Carlsbad, CA) was used. Human C/EBPβ cDNA was

subcloned into pIND at convenient enzyme cleavage sites. Constructs, containing

sense and antisense C/EBPβ sequences were verified by double-stranded DNA

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sequencing. Before transfection, pIND-C/EBPβ constructs were linearized with *Pme* I

and purified. HCT 15 cells were transfected with 5 μg of pVgRXR (Invitrogen) and

10 μg of pIND-C/EBPb using CELLFECTIN according to manufacture's instructions.

After 24 hours, cells were shifted to medium supplemented with 1 mg/ml Geneticin

5   and 10 mg/ml puromycin (GIBCO BRL) to select for transfectant clones. After 2

weeks, antibiotic-resistant cells were subcloned by limiting dilution. The expression

of C/EBPβ protein and the subsequent induction of p21$^{WAF1/CIP1}$ was determined

following induction with 10 μM muristerone A for 24 hours and Western blot

analysis. Three independent positive clones were used for all assays with essentially

10   the same results.

Representative data from clones derived from each of these cell lines is shown

in Figure 4E and F. C/EBPβ overexpression elevated p21$^{WAF1/CIP1}$ protein levels

compared to unstimulated basal levels (Figure 4E: inset). Induction of C/EBPβ also

led to an increase in the apoptotic index of these cells in both the presence and absence

15   of antioxidant. In addition, repression of C/EBPβ expression by antisense mRNA

induction nearly abolished antioxidant-induced apoptosis in these cells (Figure 4E).

Further evidence that induction of C/EBPβ mediates the effects of antioxidants on

colorectal cancer cells was demonstrated by the increased apoptotic index in response

to either 5-FU or doxorubicin in the presence of overexpressed C/EBPβ (Figure 4F).

20   In the absence of C/EBPβ overexpression, 5-FU increased the apoptotic index to 20%

whereas doxorubicin did not induce apoptosis. When these cells were induced to

overexpress C/EBPb in the presence of either 5-FU or doxorubicin, apoptosis was

increased to 70% and 80%, respectively. Taken together, these data demonstrate that the induction of apoptosis by antioxidants is, at least in part, mediated by a p53-independent induction of p21$^{WAF1/CIP1}$ via activation of the transcription factor C/EBPβ.

Another transcription factor, NF-kB, has been shown to confer resistance to

5   TNFα-mediated apoptosis, although a recent report has shown that induction of NF-kB DNA binding activity in kidney epithelial cells precedes apoptosis following serum-withdrawal. NF-kB activity can be downregulated by pyrrolidinedithiocarbamate through inhibition of the phosphorylation and subsequent proteasome-mediated proteolysis of its inhibitors (IkBs). No reduction in NF-kB

10   DNA binding activity was detected in these CRC cells at the doses of pyrrolidinedithiocarbamate used in these studies. In addition, it has recently been demonstrated that induction of p21$^{WAF1/CIP1}$ can increase NF-kB transcriptional activity, therefore it is unlikely that the antioxidant effect in these cells is mediated by a reduction in NF-kB activity. These studies demonstrate that induction of a

15   transcription factor, C/EBPβ, sensitizes CRC cells to chemotherapeutic agent-mediated apoptosis.

Activation of C/EBPβ, either directly or indirectly, induced p21$^{WAF1/CIP1}$ gene expression, leading to G$_1$ cell cycle arrest and apoptosis in two colorectal cancer cell lines. The ability of the antioxidants pyrrolidinedithiocarbamate and vitamin E to

20   induce this transcription factor, independent of functional p53, has important biological consequences on the efficiency of DNA damaging agents. Both 5-FU and doxorubicin exert their cytotoxic effects mainly through the induction of DNA

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damage. This damage, through undefined mechanism(s), signals the induction of p53,

which, in turn, leads to inhibition of cellular proliferation and apoptosis. Since

mutations of p53 occur in over 80% of advanced CRC tumors, these mutations may be

responsible for the relatively low response rate of advanced colorectal cancer tumors

5   to DNA-damaging agents, such as 5-FU. Although 5-FU is particularly successful in

the treatment of local, wild type p53, colorectal cancer tumors, the success rate falls to

15-20% in patients with advanced, frequently mutant p53-containing colorectal cancer

tumors. Thus, the ability of antioxidants (used throughout these studies at doses

obtainable in humans) to bypass the requirement of p53-mediated apoptosis

10   demonstrates the utility of combined antioxidants and chemotherapeutic agents for

advanced colorectal cancer and other solid tumors.

Example 16

Figures 5a and 5b are bar graphs of the BrDU-labelled cells (percent of total

cell nuclei) from colorectal cell xenografts derived from athymic mice treated with

15   saline, vitamin E, PDTC, 5-FU, and the combination of vitamin E and 5-FU, as a

measure of the effect of the test compound on proliferation of HCT 116 and HCT 15

cells. Figures 6a and 6b are bar chart graphs of TUNEL-positive cells (percent of

total cell nuclei) also from xenografts derived from athymic mice treated with saline,

vitamin E, PDTC, 5-FU, and the combination of vitamin E and 5-FU, as a measure of

20   the effect of the test compound on apoptosis. Tumor tissues were fixed overnight in

4% (v/v) paraformaldehyde and embedded in paraffin according to standard

histological procedures. Sections were pretreated with 10 mM citrate buffer (pH 6.0)

and incubated with PC10 monoclonal antibody against BrDU (Boehringer

Mannheim). TdT labeling of fragmented DNA (TUNEL) was performed according to

manufacturer's instructions. The proliferative index (BrDU) and the apoptotic index

(TUNEL) were estimated by the precentage of cells scored under a microscope at 200-

5   fold magnification.

Example 17

As indicated in Figure 7, PDTC treatment induces C/EBPß DNA binding

activity via a post-translational modification. (A) DKO-1 cell were treated with 70μM

PDTC for the indicated times, nuclear extracts were prepared with a [γ-^{32}P]-labeled

10   p21-NF_IL6 oligonucleotide (Lanes 1-9). Specificity assays: Lanes 10-12,

competition controls were performed on a nuclear extract derived from DKO-1 cells

treated with PDTC for 3 h (lane 5), with excess unlabeled wild-type (lane 11) and

mutant (lane 12) oligonucleotide. Lanes 13-15, supershift analyses were performed

with C/EBPα (lane 13), β (lane 14), or δ (lane 15) polyclonal antibodies. (B) Parallel

15   DKO-1 cell cultures were treated with PDTC (70 μM) for the indicated times.

Poly(A) was isolated and treatment-related variations in C/EBPβ mRNA levels were

evaluated by Northern blot analysis. IB15 is shown as a control for equivalent loading

and transfer. (C) Parallel DKO-1 cultures were treated with PDTC (70μM) in the

presence of [^{32}P]orthophosphate. C/EBPβ from cytosolic and nuclear fractions were

20   purified by immunoprecipitation from cells before (time 0) or at the indicated times

after PDTC treatment. Treatment-related variations in the localization of C/EBPβ

were analysed by SDS-PAGE followed by autoradiography or Western blot analysis

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(100 μg of total cellular protein / lane). (D) DKO-1 cells were cultured in the

presence of PDTC (70 μM) for 1 hour and then processed for immunocytochemistry

to detect treatment-related differences in the compartmental-ization of C/EBPβ

protein. In all experiments, parallel cultures treated with preimmune sera or primary

5   anti-C/EBPβ antisera that had been preincubated with *in vitro* translated C/EBPβ

protein demonstrated no fluorescent signal after treatment with the secondary Cy3-

conjugated antibody. Representative photomicrographs show anti-C/EBPβ stained

cells before and after PDTC treatment.

Example 18

10   Figure 8 illustrates the effect of PDTC on endogenous cAMP levels and PKA

activity. DKO-1 cells were treated with 70μM PDTC for the indicated times. Cell

lysates were prepared and assayed for (A) endogenous cAMP levels or (B) PKA

activity (see Experimental Procedures). The values are expressed as pmol mean per

μg protein $\pm$ s.e.m. and are representative of three experiments carried out in

15   quadruplicate.

Example 19

Figure 9 illustrates that PDTC phosphorylates C/EBPβ at Ser^{299}. (A)

Endogenous C/EBPβ from [^{32}P]orthophosphate-labeled DKO-1 cells (2 mCi/ml. 3 h)

that were treated with either 0 μM (lane 1), 70 μM PDTC (lane 2) or 50 μM forskolin

20   were immunoprecipitated with anti C/EBPβ antibodies. Labeled proteins were

visualized by SDS-PAGE followed by autoradiography. (B) Tryptic phosphopeptide

maps of *in vivo* labeled epitope-tagged C/EBPβ. Wild type (WT) and mutant (Ala^{299})

C/EBPβ, immunoprecipitated from PDTC treated or untreated DKO-1 cells with the

antibody to the FLAG-epitope, were digested with trypsin and the phosphopeptides

separated by electrophoresis and thin-layer chromatography and visualized by

autoradiography, $X_{1,2}$ were constitutively phosphorylated. The level of

5   phosphopeptide X_3 was increased after PDTC treatment in cells transfected with the

wild type, but not mutant, protein. The circle indicates the origin. (C) Comparison of

the *in vivo* phosphorylation of wild type and Ala substitution mutants of C/EBPβ from

untreated cells and cells treated with PDTC. Autoradiography (top) and C/EBPβ

immunoblot (bottom) are shown. (D) Phosphorylation of Ser^{299} within C/EBPβ is

10   essential for protein translocation to the nucleus. DKO-1 cells were transfected with

pCMV-C/EBPβ (WT) or pCMV-C/EBPβ (Ala^{299}), and treated with PDTC for 3 hours.

C/EBPβ protein was visualized by immunocytochemistry as described in

Experimental Procedures.

Example 20

15   Figure 10 illustrates that PKA phosphorylation of C/EBPβ is required for

nuclear translocation. (A) Parallel DKO-1 cell cultures were treated with PDTC (0 or

70 μM) for 3 hours. Poly(A)$^+$ mRNA and protein were isolated from each group and

treatment-related variations in C/EBPβ mRNA and protein levels were evaluated by

Northern or Western blot analysis. IB15 is shown as a control for equivalent loading

20   and transfer. (B) DKO-1 cells were treated with PDTC (0 or 70μM) or PDTC and

mPKI (myristylated protein kinase A inhibitor; 1 μM) for 3 hours. Cells were fixed

with paraformaldehyde and C/EBPβ protein visualized by immunofluorescence

staining. Treatment of cells with mPKI alone failed to induce nuclear translocation of

C/EBPβ (data not shown).

Example 21

Figure 11 illustrates that carboxymethylation of the catalytic subunit of PP2Ac

5   is inhibited by PDTC. DKO-1 cells were incubated in serum-containg media

containing [methyl-^{3}H]S-adenosyl methionine and/or 70 μM PDTC for three hours.

Cytosolic or nuclear fractions were prepared and C/EBPβ immunoprecipitated using

standard methods. Antibody/antigen complexes were resolved by SDS-PAGE and the

presece of PP2Ac was detected by fluorography (overnight). PDTC inhibited

10   carboxymethylation of PP2A subunit in nuclear fractions, and to a lesser extent, in

cytosolic fractions.

Example 22

Figure 12 illustrates that PDTC inhibits methyltransferase activation of PP2Ac.

PP2A (a and c dimer) was incubated in the presence of [methyl-3H]S-adenosyl

15   methionine, increasing concentrations of PDTC and partially purified rat

methyltransferase for thirty minutes at 37 degrees C. The reaction was terminated by

the addition of SDS-sample buffer. Samples were resolved by SDS-PAGE and the

presence of methylated PP2A catalytic subunit visualized by fluorography. PDTC

selectively inhibits the ability of methyltransferase to carboxylate the catalytic subunit

20   of PP2A in a dose dependent manner.

Example 23

To demonstrate a specific and direct inhibitory effect of PDTC on PP2A

activity, DKO-1 cells were initially treated with 17 μM PDTC for three hours. Cell

lysates were prepared and treated with the following reagents in the presence of

5 phosphorylated C/EBPβ in which the phosphate is radiolabelled, for ten minutes at 37

degrees C: I2 (a selective PP1 inhibitor), okadaic acid (a selective inhibitor of PP2A

and PP1), PDTC, I2 and PDTC, and okadaic acid and PDTC. As shown in Figure 13,

PDTC inhibited phosphatase activity in the DKO-1 extract, resulting in maintenance

of the C/EBPβ in its phosphorylated state. This effect is reversible following removal

10 of the antioxidant. This result is consistent with PDTC inhibition of the PP2A

phosphatase. In contrast, a PP1 phosphastase specific inhibitor, I2, failed to protect

C/EBPβ from dephosphorylation under the same conditions. As expected, the

nonspecific phosphatase inhibitor okadaic acid inhibited all DKO-1 phosphatase

activity, thus protecting the C/EBPβ from dephosphorylation. These results

15 demonstrate that antioxidants such as PDTC are specific inhibitors of a class of

phosphatases, such as PP2A, that are involved in the dephosphorylation of C/EBPβ.

Example 24

The effect of PDTC on cellular proliferation or apoptosis was evaluated in a

number of normal and cancer cell lines. The IC_{50} was measured as the concentration of

20 PDTC that inhibited cellular proliferation. The results are provided in Table 1. As

indicated, PDTC did not inhibit the cell growth of normal cells, but did substantially

inhibit the growth of breast carcinoma cells, gastric carcinoma cells, osteosarcoma

cells, and pancreatic carcinoma cells.

TABLE 1:Effect of PDTC on Cell Proliferation
(IC_{50} required to inhibit cellular proliferation or induce apoptosis)

5   <u>Normal cells</u>

Keratinocytes	600uM
Primary colonocytes	500uM
Primary mammary epithelia	650UM
Non-transformed rat intestinal epithelial cells	450uM

10

<u>Breast carcinoma cells</u>

MCF-7	13uM
MCF-10WT	5uM
MCF-10HRas	5uM
MDA-MB231	10uM
MDA MB-468	20uM

15

<u>Gastric carcinoma cells</u>

Hs746T	35uM
N-87	40uM

20

<u>Osteosarcoma</u>

Saos-2	10uM

25   <u>Pancreatic carcinoma cells</u>

AsPo1	70uM
PANC-1	75uM
BxPc3	100uM

30   **Example 25**

To evaluate whether antioxidants induce apoptosis in normal cells, normal and

cancerous cells were incubated with 70 µM PDTC for 24 hours and DNA

fragmentation assessed as a percentage of a control. As indicated in Tables 2 and 3,

the normal cell line (primary colonocytes) did not exhibit significant DNA

35   fragmentation after 24 hours of exposure to PDTC, whereas cancerous cells (Wild

type p53 HCA-7, HCT 116, mutant p53 HCT 15, DLD-1, and DKO-3 cells) exhibited

substantial DNA fragmentation.

**TABLE 2: PDTC Induces Apoptosis in CRC Cells but not Normal Cells *in vitro*
(I)**

Cell Type	DNA Fragmentation after PDTC Treatment (70µM) (% Control)			
	3h	6h	12h	24h
Primary Colonocytes	101±10	109±9	107±10	130±16
Wild Type p53 HCA-7	111±13	126±17	154±19	**302±35**
HCT 116	108±11	131±21	198±23	**367±49**

bold values: significantly different from untreated cells (P<0.01), as determined by AOVA

TABLE 3: PDTC Induces Apoptosis in CRC Cells but not Normal Cells *in vitro* (II)

Cell Type	DNA Fragmentation after PDTC Treatment (70µM) (% Control)			
Primary Colonocytes	101±10	109±9	107±10	130±16
Mutant p53 HCT 15	145±12	259±18	673±34	979±34
DLD-1	213±17	296±21	712±34	876±46
DKO-3	223±11	478±16	896±23	1116±54

bold values: significantly different from untreated cells (P<0.01), as determined by AOVA

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Example 26

As indicated in Tables 4 and 5, PDTC substantially reduces the toxicity of 5-FU in the murine small intestine and the murine colon. These results indicate that PDTC not only increases the cytotoxic effect of antineoplastic agents, it at the same

5   time has a palliative effect on normal cells which are exposed to cytotoxic agents.

Example 27

Isolation of C/EBPβ/PP2A methyltransferase complex

A novel multicomponent complex consisting of C/EBPβ, PP2A and methyltransferase was isolated and initially characterized. This complex appears to

10   play an important role in the regulation of PP2A and downstream transcriptional events including, but not limited to, cell division and apoptosis.

Co-immunoprecipitation techniques demonstrate for the first time that the transcriptional factor C/EBPβ is complexed with the PP2Ac protein phosphatase. This novel complex appears to play an important role mechanistically in the control of the

15   phosphorylation status of C/EBPβ by PP2A.

Additionally, the C/EBPβ/PP2Ac complex has also been shown to consist of the methyltransferase which carboxymethylates the catalytec subunot of C/EBPβ. Rat brain soluble extracts were fractionated by phenyl-Sepharose and analyzed for methyltransferase activity using exogenous PP2A heterodiver (AC complex). The

20   peak of methyltransferase activity was further fractionated by Source Q (strong anion exchange), and gel filtration chromatography. The partially purified methyltransferase in Figure 14 represents the peak methyltransferase activity from the gel filtration

column. This peak fraction of methylase activity is taken further to DEAE (weak

anion exchange) and MonoQ (a different strong anion exchange resin) columns. Both

C/EBPβ and PP2A are detectable following these additional steps. Rat brain extracts

are shown as a positive control (C/EBPβ and PP2Ac migrate at approximately 45 and

5   36 kDa on SDS-PAGE).

Modifications and variations of the present invention will be obvious to

those skilled in the art from the foregoing detailed description. Such modifications an

variations are intended to come within the scope of the appended claims.

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